

Embryo research and abortion

The British government's promised bill on embryo research, now imminent, seems bound to provoke an unwanted argument on abortion. But it is not too late to amend the bill and make it better.

ONE consequence of the British government's delay in bringing forward its promised bill to implement the recommendations of the Warnock committee on *in vitro* fertilization (IVF) and related matters is that opponents of legalized abortion have had time to organize themselves so as to seize the opportunities with which the bill will provide them. The bill will be part of the government's legislative programme for the next parliamentary session, due to be announced by the Queen next month. Meanwhile, the government has leaked news of its willingness that the time-limit for abortion should be reduced from 28 to 24 weeks of gestation. That is a sensible step, although it will not, unfortunately, satisfy the critics of abortion.

That the two issues are connected is uncomfortably undeniable, at least on a strict reading of the Warnock recommendations. The committee, faced with the need to say when it would be permissible to persist with the observation of artificially fertilized human embryos, recommended that all such research should require the approval of a new statutory body, but that in no case should embryos remain viable for more than 14 days. That time was chosen to be less than that at which, in the normal embryo, the nervous system begins to develop. Ostensibly, such an immature embryo could not be supposed to feel pain. More primitively, but excusably, the members of the committee took the view that the central nervous system is the embodiment of the soul. The obvious difficulty is that anti-abortionists are entitled to demand that naturally fertilized embryos should have the same protection as Warnock recommends.

This is a debating point, but none the less influential on that account. The underlying difficulty is that the Warnock recommendations over-insure against the abuse of human embryos by researchers. That there should be a statutory procedure by which all investigations must be approved is undisputed. By now, thanks to the experience of the committees regulating the development of the genetic manipulation of organisms, there is ample experience that statutory procedures can work efficiently and in a manner that commands the respect of researchers. What reason is there to fear that the proposed replacement of the present committee on IVF by a statutory body will be less well organized? And what reason can there be for supplementing that statutory regulation by the arbitrary requirement that no observations of an artificially

fertilized embryo should extend beyond 14 days?

The public interest that human embryos should be accorded the respect they deserve, and the Warnock committee's inclinations, could just as well be secured by requiring that the new statutory committee should be satisfied that any proposed observation on or experiment with an artificially fertilized embryo should build on knowledge already firmly established, that there is a high chance that they will yield the information expected of them and that that information, if gathered, would contribute in some substantial way to the understanding of the distinctive characteristics of human life and its preservation in a healthy state. Working with such guidelines, no statutory committee would be likely to sanction the study of a human embryo when, for example, a mouse embryo would suffice. That is why, even at this late stage, the government should scrap the arbitrary time limit wished on it by the Warnock committee. □

California lessons

Earthquake protection is plainly possible, as the Californian earthquake showed. But who else can afford it?

CALIFORNIANS are almost as taken aback that last week's earthquake should have killed fewer than 200 people as by the circumstance that there should have been a further relief of stress on the San Andreas Fault (see pages 676 and 677). In spite of the tragedy that now afflicts the families bereaved and, many more, those without housing, it is literally astonishing that an urban earthquake essentially of the same magnitude as that which killed 25,000 people in Armenia last December should have done so little damage.

The explanation is simple: California's building codes, which the state has been progressively tightening since the San Francisco earthquake of 1906. The codes have been developed so that San Francisco and Los Angeles now sport metropolitan cities' shares of tall buildings, but there has also been immense attention to detail. Just five years ago, owners of wooden frame buildings were required to bolt them to the concrete foundations on which they rest when it emerged that structures of that kind could easily be shaken off their pedestals by sufficiently strong earthquakes. The regular rehearsals of the



emergency services also did much to diminish the consequences of last week's major shake.

The most serious danger now may be complacency. The relief of stress along the San Andreas Fault at San Jose cannot but increase stresses on other locked sections of the fault. But which of them will rupture left is anybody's guess. Yet there is bound to be a tendency to suppose that San Francisco's surprising escape last week is a sign that the building codes are sufficiently stringent as they are. But they are not. Plainly, there is room for the improvement of public services, electricity and water, for example. The spectacular collapse of a mile-long section of the double-decked Interstate 880 at Oakland is a less serious problem.

Meanwhile, California's experience last week should be a lesson for others to take to heart. Tokyo and other Japanese cities, at much the same risk as San Francisco, have also been zealous in earthquake protection but will nevertheless have much to learn from last week's earthquake. But most of the other regions of the world at risk from earthquakes are, by comparison, almost unprotected. Armenia's experience last year demonstrates that, but even European countries such as Greece and Turkey are well behind California. And what is to be said of the earthquake zones of the Indian subcontinent, the south-east Pacific and the Andean zone of South America, where the primitiveness of buildings dictated by poverty seems to be the best protection? Mainland China is an even worse case, if only because seismic risks are more generally spread than elsewhere. Where are the funds to carry California's message to these places?

It would be different if methods of predicting earthquakes were in sight, but they are not, even in California. That is why the earthquake-ridden peoples of the world will sooner or later have to stomach the cost of following suit expensively on humdrum building codes. The aid community had better take note of that before too much more time has passed. □

Research by numbers

British research councils may have sacrificed too much autonomy for administrative flexibility.

THE Wellcome Trust, the British charitable foundation, has imaginatively stepped into the vacuum left by the British government's unwillingness to support the proposed study of sexual behaviour of the British population, and has offered a grant of £900,000 to allow the planned survey to be completed (see page 675). The speed of the trust's response is especially admirable. Now, the momentum of a worthwhile venture will not be dissipated. Although the immediate objective of the survey is to gather information bearing on the assessment of the risk that AIDS will spread, the survey will also throw light more generally on the sexual behaviour of a sophisticated population and, the uncertainties of surveys of this kind

notwithstanding, should provide a more conspicuous landmark than Kinsey's US studies in the 1950s. The money will not be wasted.

Meanwhile, the means by which the British government refused to back the grant deserve more attention than they have been given. The researchers responsible for the project have been working through the Economic and Social Research Council (ESRC), the smallest of the five supposedly autonomous research councils responsible for supporting basic research in Britain. Knowing that ESRC's purse was shallow, they had sought also to interest the Department of Health and the Health Education Authority, both of which are said to have been sympathetic to the proposal. But in the end, after months of temporizing, the government, apparently in the person of the prime minister herself, said no. By what right, it may be asked?

The explanation seems to lie in the agreement reached in June this year between the Department of Education and Science (DES), which handles the British science budget, and the research councils. Most of the agreement makes sense. The research councils, for example, are given the right to carry forward 2 per cent of their expenditure from one year to another. They are also free to make grants in excess of £50,000 without seeking formal approval from the DES (the limit is now fixed at 3 per cent of gross expenditure). In return, the councils agreed to a number of requirements of the government, some of which are largely formal (the research councils undertake to "increase the quality and utility of postgraduate research", for example), but some of them are intolerably irksome.

The government's interference with the proposed survey of sexual behaviour among the British thus appears to derive from the stipulation that the government should be consulted whenever research councils plan grants "liable significantly to involve Ministers including highly contentious and politically sensitive matters of moment and matters of Ministerial resolution". The same agreement says that the research councils will refer to the DES grants "interacting significantly with other domestic policies of Government, particularly if at variance with them".

Nobody will complain that the government should have a say in major international issues, continued British membership of CERN for example, but, taken together, these requirements constitute a high price to pay for the loosening of the administrative shackles that have irked the research councils for decades. As the AIDS survey has shown, the requirement is a licence for government squeamishness. In principle, it also restrains ESRC from backing research proposals designed to investigate the good sense of the Treasury's commitment to 3 Deutschmark to the pound sterling. This, for what it is worth, is a further reason for the reorganization of the research councils under a single umbrella; then it might be possible to make a stronger fight to clothe titular autonomy with some of the reality thereof. □

Adaptive optics shown on French telescope

- Theoretical image quality approached
- Development on schedule for VLT Telescope

Garching, near Munich

ON THE night of 12 October, astronomers at the European Southern Observatory (ESO) took a big step towards realizing the dream of effectively stripping away the distorting effects of the Earth's atmosphere. In a successful test of a technique called 'adaptive optics', developed by ESO with French colleagues from the Observatoire de Meudon, the Laboratoire de Marcoussis and the research centre ONERA, a dancing, bobbing image was reduced to an almost stable spot. The test also bodes well for the European Very Large Telescope (VLT), an innovative linked array of four 8-metre telescopes to be built in Chile.

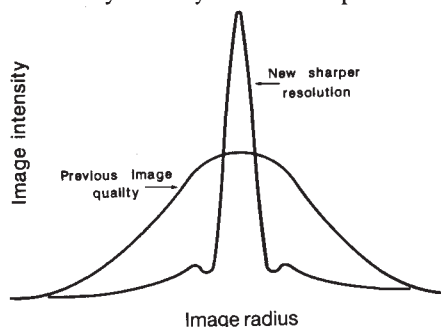
The advance is a first step towards making adaptive optics — in which secondary mirrors are physically moved to compensate for atmospheric motions that disturb a star's image — a routine feature on future optical telescopes. It has yet to be shown that the ESO system will work on any optical telescope, or that it will be as successful at visible wavelengths as it is in the near infrared. But if development goes as quickly as the ESO staff expects, adaptive optics could transform not only future telescopes but existing ones as well. ESO director Harry van der Laan said that a telescope 4 metres in diameter with adaptive optics would produce images as good as an 8-m telescope without the system.

Fritz Merkle of ESO and collaborators performed the test on a 1.52-m telescope at the Observatoire de Haute-Provence in southern France, where light pollution and low altitude translate into mediocre image quality. The new system not only took care of atmospheric distortions but also corrected for an inherent aberration in the optics of the telescope. Image quality improved slightly at visible wave-lengths and dramatically in the infrared. The best results, which came at a wave-length of 3.5 to 5 micrometres, clearly showed a diffraction ring around the star image, meaning that the image quality approached the theoretical limit for a ground-based telescope.

Adaptive optics is analogous to active optics, another technology developed at ESO and installed on the ESO 3.58-m New Technology Telescope in Chile. In both cases, a feedback loop using an electronic image analyser improves the sharpness of the images by bending a mirror. But active optics operates on a timescale of seconds or minutes, whereas adaptive

optics responds in milliseconds, fast enough to correct for the fluttering of images caused by the atmosphere. Active optics moves the telescope's primary mirror, whereas adaptive optics shunts the light from the primary mirror to a smaller mirror that can be adjusted more rapidly by small motor-driven supports.

The key to the system is a computer that



Sketch of the improved resolution achieved in the infrared using adaptive optics. The two satellite peaks correspond to the diffraction ring around the image.

processes a digital form of the distorted images and adjusts a deformable silicon mirror 1 mm thick. The adjustment improves the image before it is recorded by an electronic camera or a spectrograph. Developing special algorithms and dedicated hardware for the computer was the greatest technical problem, said Merkle.

The gamble taken by ESO in 1985 when it decided to rely on adaptive optics for the VLT seems to have paid off. Although considerable improvement is still needed, van der Laan is confident that they will be ready for installation on the VLT.

By collaborating with ESO, the French hope to add to their expertise and their reputation in the adaptive optics field. By contrast, the US military is thought to have been working for years on adaptive optics technology for the purpose of satellite tracking and laser weaponry without sharing any of its knowledge with astronomers. The most ambitious civilian programme was dropped recently by the National Optical Astronomy Observatories in Tucson, Arizona, because of lack of money and personnel.

Merkle estimates that the mirror and computer technology could be constructed from available components for as little as DM500,000 (\$270,000) per system, excluding the huge cost of software development. This is a relatively small amount in the context of ground-based telescope projects; by contrast, space-based telescopes would be "hundreds of times" more expensive. **Steven Dickman**

AIDS RESEARCH

Sex survey gets Wellcome support

London

A NATIONAL survey of British sexual behaviour, intended to provide data that will shed light on the transmission of the human immunodeficiency virus (HIV) and the spread of AIDS, will now go ahead, despite being vetoed by Prime Minister Margaret Thatcher (see *Nature* 341, 181; 1989), on the strength of a £900,000 grant from the Wellcome Trust, Britain's largest medical charity. Peter Williams, director of the Wellcome Trust, said the trustees agreed to support the study because "the results will be of great importance and of the highest scientific quality". Thatcher had refused to fund the survey because she felt it was "too intrusive", although the Economic and Social Research Council (ESRC), the Health Education Authority (HEA) and the Department of Health had supported the project.

Roy Anderson, a professor at Imperial College who helped to organize the survey, expressed great delight at the news and said the trust had acted both generously and swiftly. The survey will start in January. The research will be carried out by the department of genito-urinary Medicine at University College and Middlesex School of Medicine, the department of public health and biology at St Mary's Hospital and Imperial College, and the independent organization Social and Community Planning Research.

Detailed information on people's sexual behaviour and their attitudes to sex will, said Anderson, help to estimate the two main parameters that determine how fast, and among what part of the population, HIV spreads: these are the rate of infection and the rate of acquiring new partners. The survey will also yield valuable information about the incidence and transmission of other sexual diseases and cervical cancer.

Ben Webb

SPACE

Galileo takes off at last

Washington

ON Wednesday, 18 October, the spacecraft Galileo finally began its six-year journey to Jupiter. The space shuttle Atlantis took off after one more day of delay, due to weather, at 12.53 p.m. About seven hours later Galileo, released from the shuttle cargo bay, was steered by its own booster onto a path towards Venus. Rounding Venus and skirting Earth twice, Galileo will pick up enough velocity to reach Jupiter towards the end of 1995.

David Lindley

Erratum: THE amount of plutonium on board Galileo is not 100 kg as reported in *Nature* (341, 374; 1989). The correct amount is 50 lbs, which should be divided, not multiplied, by two, to give approximately 25 kg. □

Bay area laboratories do well

San Francisco

RESEARCH facilities at universities and national laboratories in the San Francisco Bay area fared well in the earthquake of Tuesday 17 October and subsequent aftershocks, in many cases because buildings were new or had been reinforced after previous upheavals.

The University of California at Santa Cruz, just a few miles from the earthquake's epicentre, was one of the institutions hardest hit. But because it rests on a hill above the town, the university suffered less than Santa Cruz itself, which is largely built on loose soil in the flood plain below. Residence halls were evacuated for only a few hours, and most facilities were soon functioning normally.

But the four major science facilities, including the new Sinsheimer Laboratories building scheduled for dedication later this month, suffered considerable interior damage, including chemical spills and broken laboratory equipment. By Friday, three had been declared safe, both structurally and with regard to hazardous materials, but Natural Sciences II, which houses astronomy, physics and some chemistry and biology, was still being evaluated.

Although it escaped structural damage, the Stanford Linear Collider (SLC) suffered some minor power failures, leaks in some fluid lines, and a break in a vacuum chamber. Fortunately, SLC was in the middle of a shutdown and no beam time was lost. Had the collider been running, said spokesman Michael Riordan, "the beam would have hit the wall somewhere and everything would have shut down because of the control system".

The biggest concern is that some of the 1,000 beam-guiding magnets have probably been thrown out of alignment. Next week, surveying crews will check the magnets; if too many are misaligned, the whole machine will have to be reconfigured. A preliminary laser-beam measurement has indicated that some segments of the accelerator may have shifted, although only by 100 micrometres, which is at the limit of detectability. Whether or not this is due to the earthquake is not yet known.

Stanford University itself was probably the hardest hit university in the area, and estimates damage at \$160 million. Twenty-five out of 240 major buildings have been shut, 12 of which are residence halls. Memorial Church, in the centre of campus, will be closed indefinitely. The key-stone, the critical block in the archway near the altar, has moved by more than half an inch, and the church will probably need large-scale structural repairs.

Several other buildings were damaged to varying degrees, but Stanford had

reason to be grateful for a 1988 report, *Earthquake Risk Management Report*, which paved the way for a series of structural modifications. Two older buildings, Roble Hall and the Old Pavilion, were furnished with seismic bracing just a few weeks before the earthquake.

Science facilities fared better. There was broken glass, overturned bookcases and damage to light fixtures, and some experiments were set back for a few days. But for the most part, no major equipment was destroyed. The one exception was the Keck building, the first completed facility in the new Near West campus science complex. The building itself held up well, but as much as \$30,000 worth of equipment may have been lost.

At the University of California at Berkeley, there was minimal structural damage. A few cracks appeared in various buildings, but no scientific facilities, including the seismographic station, were

disrupted. At classes were officially closed.

Above the campus, the Lawrence Berkeley Laboratory (LBL) suffered no structural damage, LBL's automatic system shut down gas valves, and a few buildings had trouble starting services up again. But otherwise, no problems were reported. All three accelerators are operating.

Lawrence Livermore National Laboratory also withstood the earthquake well. After two buildings were destroyed in 1980 by an earthquake of magnitude 5.9 with the epicentre just a few miles from the laboratory, \$25 million has been spent in seismic upgrades. This included reinforcing buildings and putting doors on cabinets containing chemicals. The most serious damage was a flood in Building 41, a laser facility, as a result of broken water pipes.

Finally, the University of California at San Francisco reported no big upsets — a few chemical spills, some cracked walls, but no significant structural damage.

Robert Buderl

The physics of destruction

San Francisco

THE fact that some areas suffered considerable damage while others escaped almost unscathed is no cause for surprise. According to Robert Page, a geophysicist with the US Geological Survey in Menlo Park, one of the major factors was "liquefaction", which occurs in sandy material that is saturated with water. The earthquake's vibration transforms such material into a slurry: because the sand particles are all about the same size, they lose contact with each other, water flows between them, and mobile near-liquid is formed. This is what happened in San Francisco's most devastated area, the Marina district, which is built largely on land-fill. The hilly areas rest on bedrock, which is much less susceptible to disruption.

In Santa Cruz, the buildings hit hardest were those of unreinforced masonry situated on the flatland in the centre of town, which lies on a flood plain. Page said a similar story probably unfolded there, exacerbated by the fact that in an earthquake ground tends to move horizontally towards rivers.

A second factor contributing to the wide variation of damage is building resonance. If a building's own natural frequency of vibration closely matches that of the driving force applied by the seismic waves, the resonance amplifies the motion, making the structure more likely to tumble. Close to the epicentre, where shock waves are of higher frequency, resonance is more likely to strike low-lying structures, explaining why, in areas such as Santa Cruz, many houses in the

flatlands were knocked off their foundations. Further away, as in San Francisco, the seismic waves are of lower frequency, and resonate with taller buildings, but in the city such structures are built to cope with earthquakes of this magnitude. They also have deep foundations that extend past the loose topsoil and into hard ground below.

Edward Wilson, a professor of civil engineering at the University of California in Berkeley who specializes in the response of buildings to earthquakes, observed that although resonance and liquefaction are important, "clearly, if you look around, the site is the most critical element". Hard sites, such as the hills of San Francisco, afford little amplification of a seismic wave; softer soil, even if liquefaction does not take place, will amplify the waves.

This probably holds true even for the Cypress section of Interstate 880 in Oakland, where a still undetermined number of people died. An upper section of the double-decker freeway collapsed onto the lower deck, because supporting columns gave way. The freeway was built in the 1950s, and the structure had been only partially strengthened since then: the cross-links that keep each deck together had been stiffened, but the vertical columns between decks had not.

Nevertheless, Wilson said, it was likely that loose underlying soil might be the crucial factor in the freeway collapse: "We had many freeway overpasses of similar design, and not all of them failed".

Robert Buderl

LOMA PRIETA EARTHQUAKE

A successful prediction?

London

TWELVE hours after last week's earthquake in northern California, the United States Geological Survey (USGS) claimed that it had "forecast" the event in a report issued last year. Californians may find the value of this forecast debatable, but the close resemblance between the earthquake and the event foretold, not just by USGS but by many US seismologists over the past decade, encourages confidence that a practical understanding of earthquakes on the San Andreas Fault is within reach.

The earthquake, estimated at magnitude 6.9 (with about one-fifteenth the energy of the great San Francisco earthquake of 1906), had its epicentre in the mountains north-east of Santa Cruz, and has been named 'Loma Prieta' after a lookout station there. Its depth is thought to be 18 km, a little more than the characteristic 10–15 km depth of microearthquakes in the region.

So far, no unambiguous surface faulting has been identified, but very steep local terrain and thick brush makes it hard to distinguish true ruptures from superficial slumps and landslides. And from such a deep focus, the fault rupture may not have reached the surface at all.

The motion was primarily strike-slip (the two blocks of crust sliding past one another), typical of the San Andreas Fault, but there was also a small thrust component (one block being forced over another). The total slip is estimated to have been about 50 cm.

Since the main shock on Tuesday 17 October at 5:04 p.m. local time, thousands of aftershocks have spread out along the 50-km segment of the San Andreas Fault that ruptured. This segment, running from Pajaro Gap in the south, near San Juan Bautista, to Lexington Reservoir in the north, is the southernmost part of the 1906 rupture, which extended north for 450 km towards Cape Mendocino.

The southern stretch has attracted the attention of seismologists since at least 1983, when Al Lindh, of USGS in Menlo Park, California, identified a 45-km section north of San Juan Bautista as being likely to rupture in the following 20 years. For 150 km south of San Juan Bautista, the San Andreas Fault is creeping gradually, dissipating the strain that would cause an earthquake, but to the north and

south of this 'central creeping segment', the fault is locked. The entire length of the 1906 rupture has been accumulating strain since 1906, making a future earthquake more likely.

Simple models suggest that when the strain relieved by a previous earthquake has been re-accumulated, the fault is ripe for another event of the same magnitude. The slip in the 1906 earthquake varied from about 5 metres near San Francisco to near zero at San Juan Bautista. The southernmost 90 km, the San Francisco Peninsula segment, slipped by only 1–3 metres, and at a continuing slip rate of 16 mm per year will recover its 1906 slip before the rest of the northern San Andreas.

Such reasoning led USGS to assign a 20 per cent probability to a magnitude 7 earthquake happening on the Peninsula segment within 30 years of 1988. But it put a probability of 30 per cent on an event rupturing only the southernmost 30 km, the Santa Cruz Mountains segment, with a predicted magnitude of 6.5. The Loma

derives from ignorance of what actually happened in 1906: estimates of the slip that occurred then vary by a factor of two. But modern geodetic instruments will give much more precise estimates of the slip that occurred last week. Moreover, the Loma Prieta event gives broad support to the idea that, at least on the San Andreas Fault, it is possible to identify 'characteristic earthquakes' — recurring ruptures of identifiable segments, with magnitudes and recurrence intervals that are, in principle, predictable.

The next important test of this hypothesis will be in the Parkfield segment, a 30-km section between the central creeping segment and the locked southern half of the fault. The Parkfield segment has experienced five moderate (magnitude 6) earthquakes since 1881, with an average recurrence time of 22 years. The most recent was in 1966, leading USGS to suggest a 90 per cent probability for a magnitude 6 earthquake on this segment in the next 30 years.

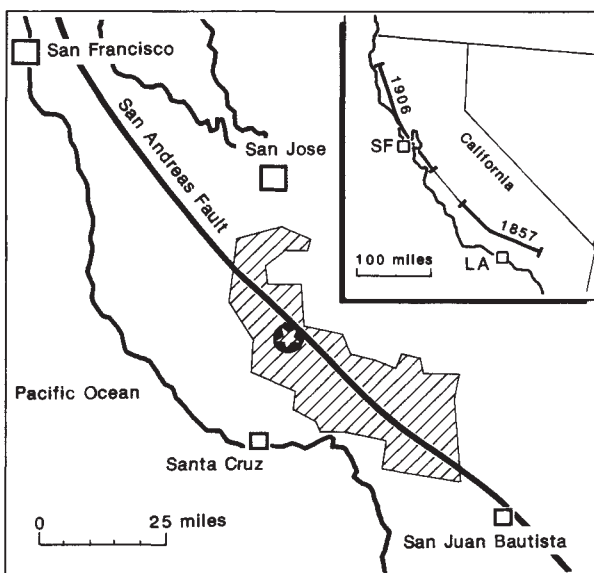
The Parkfield segment is being monitored for any precursory activity that might lead to a more specific prediction. In retrospect, there were seismic precursors to the Loma Prieta earthquake, in the form of two magnitude 5 earthquakes in June 1988 and August 1989. In each case, USGS issued a short-term (five-day) warning of enhanced probability of a larger earthquake, after which the probability reverted to its long-term value.

Similarly, there was a chance that the Loma Prieta earthquake itself could have triggered an earthquake on an adjacent segment of the San Andreas, but this threat has now receded. The largest aftershock recorded so far was of magnitude 5.2, 37 minutes after the main shock.

And what about the next one? Loma Prieta does not change the long-term probabilities for the region: there is still a probability of 50 per cent for a magnitude 7 earthquake in the Bay area over the next 30 years, and a 60 per cent probability for a magnitude 7.5–8 shock on the southern San Andreas over the same period.

But according to Scholz, the Loma Prieta rupture may have stopped 25 km short of the northern end of the region of reduced slip in 1906. An earthquake rupturing this remaining length of fault would be only of magnitude 6, but it would be much closer to the heavily populated Silicon Valley.

Laura Garwin



The San Francisco Peninsula segment of the San Andreas Fault (main map), showing the epicentre (star) and the region of densely clustered aftershocks (shading) marking the Loma Prieta rupture. Inset shows the segments of the fault that ruptured in the great earthquakes of 1906 and 1857, separated by the 'central creeping segment'. SF, San Francisco; LA, Los Angeles.

Prieta event falls between these two predictions.

Some think that USGS was too conservative. Chris Scholz, of the Lamont-Doherty Geological Observatory in New York, estimated in 1985 that a 75-km length of the segment had slipped only 1–1.4 metres in 1906, rather than the 2.5 metres assumed by USGS, leading to an earthquake recurrence time of only 60–90 years, instead of 160 years. But despite all this uncertainty, there are reasons to think that earthquake prediction is getting better.

Most of the uncertainty in current predictions for the northern San Andreas

Earthquake magnitudes

As earthquake sizes vary enormously, it is convenient to use a logarithmic scale. The well-known Richter magnitude is defined as the base-ten logarithm of the maximum seismic-wave amplitude in micrometres, recorded on a standard seismograph at 100 km from the earthquake epicentre. An increase in magnitude of one unit corresponds to a factor of 10 increase in wave amplitude, but a factor of 30 increase in earthquake energy.

France, Australia left in cold

Paris

AFTER ten days of intense deliberation, the fifteenth international Antarctic Convention Consultative Meeting came to an end in Paris last Friday still profoundly divided over the best way to safeguard the future of the continent. The 25 consultative parties and the 13 contracting parties to the Antarctic Treaty failed to achieve the unanimity required to adopt the Convention on the Regulation of Antarctic Mineral Resource Activities (CRAMRA).

CRAMRA was drafted at the initiative of New Zealand to fill a gap in the existing Antarctic Treaty regarding mineral exploration and exploitation, activities that are at present technically disallowed but not controlled. CRAMRA proposes to allow exploration, but only if stringent environmental rules are followed. Having signed the draft convention, France and Australia have since refused to ratify it, and want instead to turn Antarctica into a natural wilderness reserve and 'land of science', where all but approved scientific projects would be banned.

Two clear factions emerged in Paris, with New Zealand, the United States and Britain calling for the adoption of CRAMRA and Belgium, Italy, Greece and Austria supporting the Franco-Aus-

ROCKEFELLER PRESIDENT

Baltimore accepts, amid controversy

Washington

THE Rockefeller University announced last week that David Baltimore, director of the Whitehead Institute in Cambridge, Massachusetts, since it was founded in 1982, will replace Joshua Lederberg as president of the university on 1 July 1990, when Lederberg retires. Baltimore, a Rockefeller alumnus, says his plans include "increasing the teaching role of the university, ... bringing the Rockefeller community more sense of common purpose, increasing the role of younger scientists, and positioning the university to lead the way in understanding the nervous system".

Rockefeller's offer to Baltimore at first created controversy. Members of the faculty protested that Baltimore's links with the highly publicized misconduct allegations arising from a paper that he co-authored would damage the university's reputation. But Baltimore is said to have won over the opposition at a discussion with the faculty.

Christine McGourty



CHRISTINE MCGOURTY

At the same time, they say, the Franco-Australian proposals threaten to undermine the spirit of unanimity which has allowed the treaty to work well so far, while creating a parallel structure which, at the moment, lacks an appropriate administrative body.

But as a concession to the Franco-Australian axis, the Paris meeting recommended that a Special Antarctic Treaty Consultative Meeting be held in mid-1990 specifically to discuss the protection of the environment and dependent ecosystems. In a second recommendation, the section of CRAMRA devoted to environmental protection will also be the subject of a meeting next year.

Commander Jacques-Yves Cousteau, marine biologist and a campaigner for the idea of an Antarctic wilderness reserve, was visibly disappointed at the outcome of the meeting. "We haven't advanced one iota", he said, blaming "bureaucrats from foreign affairs ministries who think the Antarctic is their thing".

But some positive measures were adopted, including a new code of practice for the disposal of waste created by scientific missions — ironically one of the major sources of Antarctic pollution. A recommendation to control marine pollution and the creation of a new category of protected area — of 'general interest' — were also approved.

AIDS

European trials of AZT to continue

Paris

AN Anglo-French placebo-controlled clinical trial of AZT in people infected with HIV (human immunodeficiency virus) but showing no symptoms of AIDS proper is to continue despite the short-term positive results that led a similar trial in the United States to be abandoned (see *Nature* 340, 581; 1989). The trial, called Concorde 1, is jointly sponsored by the British Medical Research Council and the French Institut National de la Santé et de la Recherche Médicale (INSERM).

After the apparent success in the US trial of AZT in slowing progression to AIDS and advanced AIDS-related complex in individuals with a T4 cell-count of less than 500, the US National Institute of Allergies and Infectious Diseases (NIAID) ordered a halt to the trial. Since then, AZT (or Zidovudine in France), has been approved by the US Food and Drug Administration for use with individuals in an early stage of HIV

First woman nominated

Washington

THE deputy director of the National Institute of Child Health and Human Development, Antonia Novello, is likely to be the next surgeon-general of the United States, replacing the forthright C. Everett Koop who left earlier this year. Selection of a replacement has been slow, it is said because of the importance of finding a candidate who supported President Bush in his opposition to abortion.

Although her nomination, which would have to be confirmed by Congress, has not been formally announced by the Bush administration, Novello, a specialist in paediatric AIDS and known for her support of women's issues, is said to be the leading candidate.

Christine McGourty

Cousteau nevertheless sees next year's special meeting as a small victory and says it will give France and Australia time to prepare a more cogent proposal. He denied that France and Australia have upset the previous unanimity that allowed CRAMRA to be drafted. On the contrary, he said, the new lack of unanimity is a sign that CRAMRA should be dropped. Speaking on Saturday, he claimed that CRAMRA is, in any case, premature: "no one is currently interested in commercial mineral exploration in the Antarctic". Meanwhile, he said, nations would serve the interests of Antarctica better by tackling real threats to the Antarctic environment, posed by uncontrolled shipping and aircraft in the area.

The sixteenth Consultative Meeting will be held in West Germany in 1991.

Peter Coles

infection.

After discussions with NIAID officials, the Data and Safety Monitoring Committee (DSMC) of the Concorde 1 study has decided that "the scientific data now available do not allow strict recommendations to be made about Zidovudine treatment of asymptomatic HIV positive people based on their CD4 lymphocyte counts. This applies equally to people in trials and in clinical practice."

According to Professor Jean Dormont, of the INSERM immunopathology unit at Clamart near Paris, the US data are not in question, but allow no conclusions about AZT in asymptomatic HIV-infected individuals. "The DSMC has advised us to continue the trial in order to try to obtain longer-term results. But we will leave it to each participating centre to decide, as a matter of conscience, whether it is appropriate to put an individual onto AZT with a T4 count of 500 or not".

Peter Coles

COLD FUSION

Noncommittal outcome

Washington

THE title "Anomalous effects in deuterated metals" hardly disguised the fact that a closed meeting in Washington last week was about cold fusion. The three-day workshop (16–18 October), organized by the National Science Foundation (NSF) and the Electric Power Research Institute (EPRI), an industry-based technology development association, brought together a remarkable collection of those who have seen "anomalous effects", including Stanley Pons and Martin Fleischmann. Also present were some sceptics, notably Nate Lewis of the California Institute of Technology. Getting these people together was no easy feat: at least two outspoken critics refused to attend because they saw little in the meeting but a parade of dubious and mostly well-known evidence for cold fusion, whereas one believer had to be persuaded that the workshop was not stacked with sceptics before he agreed to attend. And, contrary to usual NSF practice, the workshop was closed to the press.

According to Paul Werbos, programme director for Emerging Technologies in NSF's division of Electrical and Communication Systems, the workshop was meant to help NSF deal with a steady stream of grant applications for cold-fusion experiments. Werbos consulted an NSF colleague on leave from EPRI, which has been supporting experiments at Texas A&M University, and the idea was born of a jointly organized meeting, co-chaired by John Appleby of Texas A&M and Paul Chu of the University of Houston, Texas.

Although some new data were presented at the meeting, a final statement issued to the press by Appleby gave no details, observing only that anomalous heating "appears to be real in many cases" and that there was reason to believe that the appearance of tritium in some electrolytic cells "is not an artefact". But others who attended the sessions said that no consensus was reached on any of the experimental results. No one denied that the detection of tritium was genuine, but several remained unconvinced that contamination had not been ruled out. And the claims of excess heat were as controversial as ever, with some saying that the calorimetry appeared to be carefully done, while others decried a lack of controls and reproducibility.

Werbos emphasized that the purpose of the meeting was not to pass judgment but to decide what research ought to be done. Both sides in the debate seem to accept that the heat measurements will probably not prove convincing one way or the other, and that the presence or absence of nuclear products is the crucial evidence. To this end, Edward Teller of Lawrence

Livermore Laboratory suggested that partial replacement of palladium with uranium-235, which accepts neutrons readily, would help to determine if some kind of neutron transfer initiated by deuterium was at work.

In about a month, the proceedings of the NSF/EPRI workshop will be released, along with some recommendations for further research. This report should appear at about the same time as the conclusions of the Department of Energy's (DoE) special panel on cold fusion, a preliminary version of which recommended against putting any money into the area (see *Nature* 340, 174; 1989).

Thomas Schneider of EPRI did not

accept the idea, put about by critics, that the purpose of last week's meeting was to provide a counterweight to the anticipated negative verdict of DoE's "killer commission", as John Bockris of Texas A&M has dubbed it.

But if the aim was to find some common ground between believers and critics, the workshop had little success. Those who seem able to get anomalous results see a need for further research, and argue that one should not dwell on "negative" results because in those experiments, evidently, the conditions are not quite right for cold fusion to work. Critics, on the other hand, maintain that if you are allowed to keep positive results and throw away the rest you can never be proved wrong: it becomes, as one sceptic put it, religion, not science.

David Lindley

GENOME MAPPING

Speak softly or carry a big stick

Washington

FUELLING the debate over how to coordinate and finance international efforts to sequence the human genome, James Watson, director of the National Institutes of Health Center for Genome Research, said last

Watson confirmed these fears, saying it was possible that the United States would do all the basic research while others would focus on the commercial applications.

Although the United States could map and sequence the human genome alone, said Watson, "it makes sense to reduce the cost to the American public by some form of sharing". And a political reason for collaboration, Watson added, was that if the United States were to tackle the project alone, the prospect of one country claiming all rights to the data might put the rest of the world "ill at ease".

But instead of a multitude of bilateral agreements — such as the agreement between the UK Medical Research Council in Cambridge and Washington University Medical School in St Louis to sequence the nematode — Watson endorsed HUGO (the Human Genome Organization) as the vehicle to facilitate international collaborations. Although HUGO has been accused of inactivity, leading some to argue against contributing to it, Watson says supporting it will save the United States "a great deal". If other countries did not participate, he said, he would hold on to the US data "for quite a bit of time".

HUGO treasurer George Cahill, of the Howard Hughes Medical Institute (HHMI), said Watson was "playing hardball" and that he would favour "a more soft and more diplomatic" approach to encourage international genome mapping efforts. More countries want to join HUGO, he said, and will do so when it has "more stability and funding". Cahill says HUGO may begin to move more quickly within the next few weeks after it becomes a US corporation, which would allow it to receive a \$1 million gift that HHMI is considering. After that, "we could really have some clout and could get moving", says Cahill.

Christine McGourty



week that the United States should ensure that other countries support basic research by threatening to restrict access to its data. Testifying at a hearing of the House of Representatives subcommittee on international scientific cooperation, Watson declared that "America has been subsidizing the rest of the world for too long in science and it's time that [others] came in".

The subcommittee, set up three years ago to examine financing for 'big science' projects, is scrutinizing the \$3,000-million human genome project because of fears that the United States might end up subsidizing foreign biotechnology industries.

Readmission with strings

Athens

THE World Psychiatric Association (WPA) last week voted to readmit the Soviet All-Union Society of Psychiatrists and Narcologists to conditional membership. But despite the overwhelming vote in favour (291 for, 45 against and 19 abstentions), the readmission was not smooth.

The vote was plainly a consequence of tough bargaining. During the meeting, the All-Union society read out a statement acknowledging that there had previously been psychiatric abuses for non-medical, including political, reasons.

Western human rights campaigners, notably the International Association on the Political Use of Psychiatry, described as largely cosmetic the reforms introduced in the Soviet Union in the past five years, when patients' rights to appeal against involuntary hospitalization have been strengthened, the 'special' psychiatric hospitals have been transferred from the prison service to the Ministry of Health and open discussion of Jung and Freud has been allowed.

In particular, the British Royal College of Psychiatrists was still, last week, seeking a formal retraction of the Soviet charge of slander, while WPA itself was criticized for having failed to put forward an application for membership from the Independent Psychiatric Association, the unofficial alternative to the All-Union society.

In the event, the unofficial body was granted unconditional membership, but the All-Union association will be monitored continuously by the WPA review committee; it was warned, last week, that the continuation of abuses may lead to another expulsion. The Bulgarian and Czechoslovak associations returned almost unnoticed to the fold, but the Cuban association, whose government has little time for *perestroika*, did not apply for renewed membership.

The readmission of the Soviet association to the club was the decision of the WPA General Assembly, which spent much of last week wrestling with ethical issues. Should the suicide of patients be considered 'rational'? What are the rights of third parties providing funds, governments or insurance companies, in the therapeutic process? And what if a genetic basis is established for psychiatric illness? Sir Martin Roth of the Cambridge Clinical School, argued in his closing address that the benefits of such a facility would be offset, at least in part, by the dangers of abuse. Roth particularly emphasized that awareness of the genetic background of psychiatric illness should not lead to prejudice against the carriers of these genes.

Vera Rich

Death-knell for LD50?

London

BELIEVING that a more humane alternative now exists, the European Commission has decided to put a stop to the assessment of the toxicity of chemicals by the present LD50 (lethal dose — 50 per cent) criterion, in the determination of which large numbers of animals are killed. The criterion is at present given statutory backing by European legislation dealing with the safety of medicines and the introduction of new chemicals into the environment.

After a two-day meeting in Brussels, a senior Commission spokesman said the LD50 system, which has been used since the 1920s, should be replaced, after a £500,000 validation programme, by a "fixed-dose" criterion. Dr Michael Balls, from the University of Nottingham Medical School, said the meeting had ended in a mood of great optimism. He applauded the Commission's decision and said that other countries in the Organization for Economic Cooperation and Development (OECD) now using the LD50 criterion should adopt the proposed new system.

LD-50 trials, in which varied doses of chemicals are given to groups of animals so as to determine what dose kills half of a group, have long been condemned by animal rights groups. The criterion was adopted in 1985 by the OECD countries, but in 1987 the guidelines were changed to

reduce the number of animals needed, although the principle remained the same.

Balls, who is also a trustee of the Fund for the Replacement of Animals in Medical Experiments (FRAME), says that the more efficient fixed dose formula would reduce the use of experimental animals. "A good toxicologist can administer a fixed dose, depending on what is known about the drug, and judge the toxicity of a chemical by the animal's response", he said.

The validation studies on the fixed-dose criterion, initiated by the UK Home Office and Department of Health with the European Commission, have been carried out in 31 laboratories in Europe, Japan and the United States. At the Brussels meeting, the Commission was presented with results demonstrating the feasibility of the fixed dose procedure. According to Balls, a senior Commission spokesman said he was "convinced" that the method would work and that the Commission was "determined" to see it put into practice.

The Commission must now persuade the OECD countries outside Europe to accept the new guidelines; Sweden and Switzerland gave their approval at the meeting. "The European Commission has thrown down the gauntlet to the US and Japan". Balls added: "It is no longer a question of science, it is now up to the politicians".

Ben Webb

Laboratory directors fight bureaucrats

New Delhi

A NEW ruling by the Council of Scientific and Industrial Research (CSIR) requiring the directors of its approximately 40 laboratories to quit after six years of service has thrown the agency, India's main research organization, into a crisis. One director has resigned, some are planning to take CSIR to court and most are rebelling against their director general, A. P. Mitra.

The provocation is a letter from CSIR headquarters in New Delhi informing the directors that under revised rules they have the option, after a non-renewable term of six years, of remaining as 'director-level' scientists or moving to CSIR headquarters.

Implementation of the rule comes two years after it was recommended by a review committee; CSIR laboratory directors protested at the time and hoped the new rule would not be put into practice. But their protests seem to have been in vain.

Enforcement of the six-year rule would require 20 of the 40 directors to leave their posts by next year. P. K. Ray, who resigned a \$60,000-a-year US job to become director of the industrial toxicology research centre, is one of many who have been asked to sign a

new contract. So far only one has complied.

Some argue that the new letter is in breach of the previous contract, which did not specify a fixed tenure. Three directors are planning to fight CSIR in court and six are hoping to return to institutions which they left to join CSIR. P. K. Jena, director of the Regional Research Laboratory (RRL) in Bhubaneswar, resigned in disgust, and J. N. Barua, director of RRL in Jorhat, has refused to vacate his post although he has completed six years.

Pushpa Bhargava, director of the Centre for Cellular and Molecular Biology, has asked Prime Minister Rajiv Gandhi to intervene but with general elections due in November, Gandhi has little time to defuse what could be the biggest crisis in CSIR's 40-year history.

The directors say the crisis could have been averted had Mitra consulted them beforehand. He is also accused of favouritism in giving 'courtesy extensions' to five directors who have completed six years, while asking others to comply with the rule. But Mitra seems unperturbed. He has avoided meeting the directors or the press.

K. S. Jayaraman

New advisory board approved

Washington

Louis Sullivan, Secretary of the Department of Health and Human Services, surprised the biotechnology research community two weeks ago with the announcement that he was about to give his formal approval now to the establishment of a new government advisory body on biotechnology, to be called the National Biotechnology Policy Board (NBPB). It will concentrate on technology transfer from university and federal research laboratories and the competitiveness of the US biotechnology industry.

Congress directed the National Institutes of Health (NIH) to establish the board last year and wanted to see its first report in January 1990, but members of the board have not yet been selected from

Britain regulates organism release

London

REGULATIONS put before the British Parliament two weeks ago will make it compulsory, from 1 November, to notify the government's Health and Safety Executive (HSE) of the use or deliberate release of genetically manipulated organisms. HSE must be given 90 days' notice to allow time for a safety assessment of any proposed activity.

The new scheme, first put forward in 1987 by the Health and Safety Commission (HSC) and its Advisory Committee on Genetic Manipulation (ACGM), replaces the present voluntary system of notification. Richard Clifton, chief administrator of the executive's medical division, said the changes were necessary to keep up with advances in the field. He said the HSE had adopted a "pragmatic" and "step by step" approach to safety regulation.

The new provisions also require that laboratories assess the risks involved using a method approved by the HSE and that a genetic manipulation safety committee be set up at each centre undertaking such work. The regulations revise the definition of 'genetic manipulation' to include the incorporation of heritable material into an organism either directly or indirectly, using vector systems, and also provide for simplified annual retrospective notifications for low-risk activities.

In due course, the regulations now imminent in Britain may be overtaken by impending legislation. The European Commission has yet to agree the form of a directive, which may well be more stringent, while there is every likelihood that the British government's expected bill on environmental matters will also deal with the release of engineered organisms.

Ben Webb

a list of about 80 candidates. Responsibility for establishing it lies with the NIH Office of Recombinant DNA Activities, whose director, Nelson Wivel, says that it might be in place early next year, now that Sullivan is ready to sign the charter.

The board will consist of representatives from all the federal agencies that support or regulate biotechnology research, four university researchers, four representatives of the biotechnology industry, two members from state biotechnology development programmes, one member of a charitable institute and a bioethicist.

The Congressional Bioethics Board (CEB) was charged with reviewing reports from the NBPB, but is unlikely now to do so: after a short and controversial life, the CEB was dissolved on 1 October (see *Nature* 341, 6; 7 September 1989). It made no progress on any of its planned studies, the board members spending most of their time arguing over the selection of members for its advisory committee, with a candidate's views on the abortion issue being the deciding factor. Bob Cook Degan, former executive director of the board, says that for the moment he thinks the board is "dead permanently. It's certainly dead transiently".

Some of the NBPB's responsibilities, such as its mandate to "enhance basic and applied research", might overlap with those of the existing Biotechnology Science Coordinating Committee (BSCC), yet another advisory body in this field which has likewise not been without its share of controversy. The president's Office of Science and Technology Policy set up the BSCC in 1985 to coordinate the regulation of biotechnology across all the federal agencies. Last year it became bogged down in a dispute between the Environmental Protection Agency (EPA) and the Food and Drug Administration over whether the EPA should regulate non-coding as well as coding genetic sequences. An EPA rule on the regulation of genetically manipulated microorganisms was delayed. Last week, Bill Reilly, the EPA administrator, said it would be ready early next year.

But a report published two weeks ago, written by Sidney Shapiro of the University of Kansas for the Administrative Conference of the United States, a Washington-based think-tank, is highly critical of the BSCC and recommends that it should be dismantled. BSCC's chairman, John Moore, who is also a deputy director at the National Science Foundation, says the committee is at present discussing how the new NBPB will affect its role. But he says that although some of the criticisms might have been valid in the past the BSCC is "on a pretty good keel right now".

Shapiro says that gaps still exist in the

Microinjection patent granted

Washington

THE US patent for microinjection, the most widely used technique for creating transgenic animals, was granted this month to Thomas Wagner of Ohio University and Peter Hoppe of Jackson Laboratories in Bar Harbor, Maine. The patent is not restricted to any particular species of mammal or the transfer of any specific gene.

Wagner and Hoppe have assigned the patent to Ohio University, which has licensed the commercial rights to the small biotechnology company DNX Inc. University researchers can continue to use the technique free of charge, but biotechnology companies that wish to make use of microinjection for research purposes will have to pay an annual licence fee. Companies wanting to use the technique to create commercial products will be able to obtain an exclusive or a non-exclusive licence, which will be negotiated on a case-by-case basis.

Holtzman estimates that about 35 companies are now using microinjection; its applications include the development of the multi-million dollar-clot-dissolving drug TPA (tissue plasminogen activator), and of Factor V111, the blood-clotting protein used to treat one form of haemophilia. It was also used to develop the 'oncomouse', the first patented transgenic animal, which is being sold by Dupont and is expected to be useful in the testing of drugs against cancer. Wagner has assigned his royalties to the university's Edison Animal Technology Center.

Wagner and Hoppe filed for the patent in 1981, shortly before they published an account of the research in which they were the first to achieve, in a single experiment, the introduction of a foreign gene into a different species of mammal and its transmission through the germ line into a second generation of animals. Although microinjection was being used by other researchers at the same time, no competing patents are thought to have been filed and Stephen Holtzman, Ohio University's vice-president for corporate development, says the patent is "strong and enforceable". Ohio University has also filed for the patent in Europe.

Christine McGourty

regulation of genetically manipulated organisms and that the existing biotechnology regulation framework "is a prescription for inconsistent regulation and litigation". He recommends that a new committee be set up with a broader membership and permission to consider a wider range of issues. It should be more accessible to the public. Margaret Mellon of the National Wildlife Federation says that Shapiro's criticisms are not out of date, and that the burden lies with the BSCC to prove that it is not a "creature of industry".

Christine McGourty

Conflict of interest

SIR—There was one omission in your leading article on conflict of interest (*Nature* 340, 664; 1989). This is the situation when an academic department, or individual, receives both governmental and industrial funding. The conflict arises not from individual inclinations towards the source of the funding, but rather from the source of the funding itself. Benefits, if any, from research supported by the government should go back to the ultimate source of the funding, the taxpayer. On the other hand, benefits from research supported by industry end up as profits to shareholders.

The conflict arises because in many cases the benefits are not the same. I think we would all agree that industrial money becomes available to an academic department or individual because of a good track record in the fruition of the research which in most cases was supported by government funds. In other words, industry buys rather cheaply the fruits of government funding. Shareholders thus get a pay-off from this latter work, while taxpayers pay twice, once for supporting the original research and again in paying for the results of the research in the form of profits to the company.

One remedy is to cut off all government funding to departments or individuals who obtain industrial funding. This move will not, however, solve the main problem. In our capitalistic society, the only solution is for a portion of the profits resulting from research supported by both funding sources to be ploughed back by industry, either into general government research funds or back to the taxpayer in the form of payback into general government funds. Equity demands that research supported by government supposedly to benefit all should not be used to benefit only a few. Just because industry is the only source, for example, of mass-produced drugs does not mean that industry should not pay a fair share of the taxpayer's part of the cost of the research done to obtain these drugs.

PHILIP SIEKEVITZ

Rockefeller University,
New York, New York 10021-6399, USA

SIR—Your sensible leading article (*Nature* 340, 664; 1989) missed one important point. Rules about conflict of interest are designed to eliminate unfair personal gain, or at the least to minimize its likelihood. In the leading article and in most other discussions of conflict of interest, personal gain is assumed to be of the financial or other material variety.

Everyone is to a greater or lesser degree interested in material rewards. But some scientists, perhaps particularly those who are the brightest and most ambitious, are

likely to value academic reputation and prestige at least as highly as financial gain. Scientists may therefore be susceptible to the temptation to act in ways that may enhance their reputation or may damage the reputations and research opportunities of academic competitors. The obvious situation in which this may occur is in peer review of papers or grant applications.

Conflict of interest rules that concentrate purely on material issues are likely to miss many situations in which scientists can take unfair advantage. Guidelines should also require scientists to be explicit about purely academic conflicts of interest. I suspect that many of the complaints about unfair refereeing would disappear if this were the case.

DAVID F. HORROBIN

Efamol Research Institute,
PO Box 818, Kentville,
Nova Scotia, Canada B4N 4H8

Alkaline rain?

SIR—Søren Peter Lauritz Sørensen (1868–1939) must be turning in his grave in Copenhagen. As every student of introductory chemistry knows, Sørensen (in 1909) defined pH as $-\log a_{H^+}$, and therefore, as acidity increases, the pH value decreases. Although David Swinbanks' report on acid rain in Japan (*Nature* 340, 671; 1989) clearly and consistently recognizes this fact, the title "China blamed for high [sic] pH," apparently written by someone other than the author, is incorrect and does not make sense.

GEORGE B. KAUFFMAN

Department of Chemistry,
California State University, Fresno,
Fresno, California 93740, USA

To boldly go . . .

SIR—The article by Andrew Tudor on the image of scientists in horror films (*Nature* 340, 589; 1989), was amusing and interesting. I'd like to add a note about more recent films in the genre of science fiction.

Lately there has been a reaction against the *Frankenstein* image. Films such as *Altered States* and *Brain Storm* have begun taking a rebellious, almost faustian tack, in which the scientist is depicted as an adventurer, a hero. Even if his daring leads to pain, that is nobody's business as long as the only one hurt is himself. Since biblical times there have been messages decrying hubris, and *Frankenstein* does warn us to beware unforeseen consequences. Still, it's good to hear the other side as well.

The best recent example of these two views in conflict were two popular films in the *Star Trek* series. In *The Wrath of Khan*, the son and former wife of Captain

Kirk have invented a 'Genesis Device' which causes life-bearing worlds to coalesce out of a dusty nebula. This unabashedly faustian film utterly rejects the biblical-Frankenstein code, depicting this creative act as a glorious one, filled with hope and pride.

But old habits reassert themselves in the sequel, *Search for Spock*. The genesis world is flawed, falling apart just as Frankenstein's monster did. Indeed, the 'creator' is killed by his creation. The lesson, once again, is that man should never try to assert the prerogatives of heaven, and what was noble in the earlier film is now portrayed as horrible.

All this may be dismissed as irrelevant, but it is in popular media that public attitudes — and our children's — are formed. Scientists may stay aloof, but that will surely leave myth-making in the hands of ignorant men.

DAVID BRIN

11625 Montana Avenue, Apartment 9,
Los Angeles, California 90049-4676,
USA

Plagiarism

SIR—The view of the National Institutes of Health Investigating Committee as reported in *Nature* (340, 173; 1989) is surely mistaken. Plagiarism is not "at least as serious a misconduct in science as outright fabrication of experimental data". Both fabrication and plagiarism are dishonest and abuse the peer-review system, but fabrication is harmful for science in a way that plagiarism is not. Fabricated data can be detected and corrected (if at all — consider the difficulties in ecological research) only at great cost, whereas plagiarism is often a matter of public record. When undetected, fabricated data are actively misleading, whereas plagiarism merely introduces redundancy. Neither is excusable, but they are not equally damaging to science.

IAN J. GORDON

Department of Zoology,
University of Nairobi,
PO Box 30197, Nairobi, Kenya

Mirror in space

SIR—In response to the suggestion of putting a large lightweight mirror in space between Sun and Earth in order to reduce the solar radiations on our planet (*Nature* 340, 603; 1989), let me point out that the energy requirement for such an operation would be substantially diminished (two orders of magnitude, at least) if we could take the Moon as a starting point, that is, if we managed to build the mirror in the Moon with lunar material and subsequently bring it to the appropriate point.

KEPA ZUBIA

Unbemendi, 113, Apartado 829,
48080 Bilbao, Spain

The future of research in medicine

John Galloway

The government intends to reform Britain's National Health Service. Medical research — much of which is supported by charities — will suffer if the government does not think again.

NO ONE with any sense disagrees with the British government's aim to make the country's National Health Service (NHS) more effective. It is ironic therefore that its proposals for re-organization laid out in the White Paper *Working for Patients*, a statement of the government's intentions published earlier this year, may destroy the principal means by which that aim can be achieved. Good quality information and its efficient dissemination are the routes both to better services and to greater value for money. Research is the activity that produces new and better information; and research is probably what is at greatest risk from the government's plans.

Role of charities

The White Paper is sparing — to say the least — in its consideration of science. The paragraph that starts so promisingly on page 38 ("The Government is firmly committed to maintaining the quality of medical education and research . . .") provides no evidence that it understands the critical part played by medical research in the evolution of the NHS or of the effect that reform will have. Nor does it appear to appreciate the extraordinary role in the development of medical practice — prevention, treatment and support — played by medical research charities and trusts in partnership with the health service. Indeed Working Paper 2 (paragraph 4.12) is less than reassuring on this final point — "Medical charities and foundations expect the NHS to fund in full or in part the service support of research they carry out". There is no hint whether the future health service will continue its half of the partnership and provide this service support or not.

Also on page 38, there is the promise "to discuss with Regional Health Authorities (RHAs) how best to ensure that other costs associated with training and research are also met without putting one hospital at an unfair disadvantage by comparison with another". Again, there is no clue as to whether considerations of fairness will apply to the health service's charitable partners in many crucial medical areas.

It is a curious feature of British medical research (almost certainly a unique one) that as much research in Britain's universities, medical schools, hospitals and research institutes is financed by independent charities and trusts as by the government. Table 1 brings a number of features of the British system into sharp relief.

Spending by the members of the Association of British Pharmaceutical Industries (ABPI) is comparable with the total expenditure of the Advisory Board to the Research Councils (ABRC) or that of the University Funding Council (UFC). Presumably this is an indication of the importance placed on research by the pharmaceutical industry — or the relative lack of it by the government.

More to the point, total spending on medical research from government sources is less than 1 per cent of the cost of running the NHS. This figure contrasts with the funds provided by the members of the ABPI, who spent an estimated £743 million on research and development during 1988 (close to 15 per cent of the value of their sales of £4.8 billion in the same year). The greater part of government spending on medical research is covered by the £150 million or so grant-in-aid to the Medical Research Council (MRC). This figure is now almost exactly matched by the members, and the handful of affiliate members, of the Association of Medical Research Charities (AMRC) (Table 2 — see over).

Such a large contribution from charities appears to have no parallel in any other research arena. Nor does any other country comparable with Britain in its engagement in medical research depend to such an overwhelming extent on charitable support. This support could be threatened by present government plans in its intended reforms of both the universities and the NHS. The spectre of the necessity of paying general university overheads has appeared (and remains) for those charities supporting university

research through grants. The prospect is a grim one for charities and scientists alike; the level of medical research supported through grants from the charities could be halved at a stroke. The outlook within the health service, as opposed to the university and polytechnic systems, may be even worse. At least within university settings the framework for research will remain. Within the reformed health service it is unclear that it will.

Most of the charities are 'mission orientated'. Their interest is in a single disease, cystic fibrosis or muscular dystrophy for example, or in a group of closely related diseases such as the leukaemias or the cancers generally. Often these charities are the only source of money for research into their chosen disease, or at the very least the principal source (although there are notable exceptions; whereas some diseases enjoy great popularity with the public, others do not, sexually transmitted diseases or psychiatric illness for example). There are few charities or trusts with a broader, more philanthropic interest in medical research (the Wellcome Trust is the best example).

Cancer research

Cancer research is the clearest example of charitable support of research in medicine (Table 3). Government sources account for only about 10 per cent of the £100 million a year spent on such research, currently about £2.5 million through the Department of Health itself and another £9 million by the MRC. The latter figure has fallen steadily, from 11 per cent in 1985–86 to about 7 per cent of the MRC's total research expenditure in the present

TABLE 1 Research funding in Britain, 1989

Funding body	£(million)
Department of Education and Science (DES)*	
University Funding Council	780
Polytechnic and Colleges Funding Council	10.3
Advisory Board to Research Councils	816
(includes Medical Research Council)	(150)
Health Department	
Health Services Research	29.5
Non-department bodies funded through Health Department budget	
(NRPB, NIBSC, PHLS, CBLS) [†]	54
Association of Medical Research Charities	148
Members of the Association of British Pharmaceutical Industries	743
Industrial and commercial spending in universities and polytechnics	260

*Total spending by the DES through the University Funding Council and the Polytechnic and Colleges Funding Council is £6,000 million.

[†]National Radiological Protection Board, National Institute of Biological Standards and Controls, Public Health Laboratory Service, Central Blood Laboratory Service.

year. The other 90 per cent is provided by charities — the Imperial Cancer Research Fund (ICRF), the Cancer Research Campaign (CRC), the Leukaemia Research Fund (LRF), all large national charities, together with numerous smaller charities, many of them locally organized and supporting research in particular geographical areas or even limiting spending to individual hospitals.

Even these figures, telling as they are, hardly reveal the extent to which, in Britain, responsibility for cancer research and the development of clinical oncology as an arm of cancer medicine lies with the charities and depends on the initiatives they take. The Cancer Research Campaign has established and finances academic departments of clinical oncology in the Universities of Glasgow, Manchester, Liverpool, Cambridge, Southampton and London (at the Institute of Cancer Research, allied to the Royal Marsden Hospital). In Nottingham a new department of clinical oncology is being set up by the CRC collaborating with the Regional and District Health Authorities. Clinical Oncology Units have been established by the ICRF at St Bartholomew's Medical College, London, and the Universities of Edinburgh and Oxford. Indeed, only one chair of clinical oncology in Britain is not the result of an initiative by one of the biggest cancer research charities and its endowment by private subscription. The Leukaemia Research Fund has endowed chairs of haematological medicine in Cambridge and haematological oncology at the Royal Postgraduate Medical School. The record of the British Heart Foundation is even more impressive with 14 endowed chairs of cardiology, and subjects closely allied to it, distributed throughout Britain, and six personal chairs.

These few examples are not, nor are intended to be, exhaustive of the degree to which the charities provide one vertex of a medical care and research partnership triangle — the other two vertices being the health service and the medical schools. But they provide the clearest instances.

Specialist treatment

The key characteristic of most medical research charities is that the basic research they support should be turned into genuine benefits for those suffering from the particular disease, and for their families. The development and support of specialist treatment units is a large part of the activities of many charities. The Cystic Fibrosis Research Trust (CFRT) is a good example. The trust's main activity is research, into the nature of the faulty CF gene and into heart-lung transplants for example. Recognizing that those with the disease fare better in specialist CF centres, the CFRT finances clinical fellows, in the shape of registrars reading for medical

TABLE 2 Expenditure on research by the Medical Research Council and Association of Medical Research Charities

Year	MRC £ million	AMRC £ million
1983–84	107.5	76.8
1984–85	117.1	86.6
1985–86	122.3	108.8
1986–87	128.3	110.2
1987–88	139.8	137.6
1988–89	149.0	148.2

Ostensibly MRC spending has risen by 40 per cent, AMRC spending by 93 per cent. But in reality MRC spending has probably been falling for some years; what looks like an average rise of about 8 per cent a year is in real terms a fall of up to 3 per cent a year. AMRC spending, by contrast, is rising in real terms, probably by about 10 per cent a year.

TABLE 3 Principal sources of support for cancer research in Britain, 1987–88

Charity	Contribution (£million)
Imperial Cancer Research Fund	38.8
Cancer Research Campaign	24.6
Medical Research Council	9.9
Leukaemia Research Fund	6.3
Ludwig Institute for Cancer Research	3.0
Institute of Cancer Research	2.0
National Health Service	2.3
Yorkshire Cancer Research Campaign	1.7
Tenovus	1.4
North of England Cancer Research Campaign	1.0
Total	91.0

Grants — often with large capital components — totalling more than £7 million were awarded by the Kay Kendall Leukaemia Fund between 1986 and 1988. Research spending for the current year is probably at a considerably higher rate for most charities, estimated at £47 million for the Imperial Cancer Research Fund and £37 million for the Cancer Research Campaign.

degrees at CF treatment centres (14 at the last count).

Specialist centres are often supra-regional, but might equally be placed in district hospitals. There are Muscular Dystrophy Group medical centres at Newcastle and Hammersmith that have received considerable support from the charity for 12 and 15 years respectively. The two centres have established excellent reputations and patients are referred to them from most of Britain. The centres carry out research but also offer clinical diagnosis and management. Substantial and long-term commitment from a charity to a centre within an existing department has enabled the development of a particular area of clinical research and care.

These last examples illustrate one simple way in which the government's attitude to the health service is damaging research and could damage it further. An

immediate consequence of chronic underfunding is the diversion of research funds to routine clinical care which must then result in a decline in the level of clinical, as well as biomedical, research. Many of the medical research charities expected that their experimental initiatives in turning research into treatment would in time become part of the health service routine. This is now not happening. Charities are, of necessity, shouldering the responsibility for the long-term provision of treatment. Indeed, the government would probably like to turn the screw even further and put pressure on charities to take over responsibility for government initiatives, cancer prevention for example, as was clear from informal discussions between the CRC and the junior health minister last year.

Innovation in care

Medical research aims at innovation in health care. First-class clinical research facilities are essential to evaluate new methods both of treatment and management before they are introduced into routine use. It is difficult to imagine any better means of demonstrating the benefits (discarding those of no value) of innovation — in the short and the longer term — except through expert centres working individually or, preferably, in co-operation. These are exactly the sort of centres that CRC and ICRF have set up within the cancer field.

One other way of evaluating new treatments is simply by pooling the experiences of many different hospitals, whether or not they represent centres of research excellence. The multi-centre clinical trial or study is often the key to the evaluation of new treatment, leading to identification of unwanted effects which in turn lead to modification and improvement or to the treatment being dropped.

The government is proposing that individual hospitals will have the right to 'opt out' of central control and become self-governing. The fragmentation that will inevitably follow cannot but make the collection of information more difficult, whether as part of clinical trials of treatment, or for other purposes.

It is hard to imagine, for instance, that a study like that by Wariyar and Richmond (*Lancet* i, 387–388; 1989), on the relationship between low birthweight and disability, would be feasible outside an integrated health service. Such studies, which depend on centralized records, become increasingly important as medical care keeps more premature babies alive, some of whom will be disabled. Sadly, to the social and financial costs of neonatal intensive care must be added those of life-long disablement. Even within an integrated health service it was difficult to follow up the disabled children; a 4 per cent disability rate among those "easy to find

and review" has to be compared with a 35 per cent rate in the difficult groups.

Problems of fragmentation have already hit perinatal medicine in the NHS. The number of sets of triplets born each year has virtually tripled with the emergence of private *in vitro* fertilization clinics. A single set of triplets monopolizes the intensive-care facilities of the average neonatal unit. Conceived profitably by private medicine, the problems of neonatal care for the babies falls on a health service short of money. And, of course, the chances of babies not surviving (or if they do survive, of being disabled) increase with the multiplicity of the birth.

Indeed, the ability of modern medicine to prolong life, often for very long periods, demands thorough long-term follow-up of those treated. Quality of life, rather than simply survival, becomes the overriding concern. Furthermore the long-term effects of treatment begin to make an appearance and offer evidence for one form of treatment rather than another. Perhaps nowhere so much as in cancer medicine have the two issues assumed more importance. Recently, for instance, a paper in the *British Medical Journal* (298, 1611; 1989) described the long-term follow-up of women with breast cancer which had been treated by mastectomy and immediate post-operative radiotherapy. The study revealed the late onset — 20 years after treatment — of a heart condition and other cancers, brought on by radiotherapy, which resulted in a measurable number of deaths.

Saving money

An aspect of clinical trials that is almost entirely overlooked is that they can save money. They may well do this in the long term by demonstrating that a particularly expensive form of treatment is not effective and so can be dropped from the repertoire. But even during a trial, half the patients may not receive an expensive treatment they would probably receive if they were not entered in the trial. A good example is the European trial of carotid endarterectomy, an operation of as yet unproven benefit, whose realistic total cost is probably about £3,000 in Britain and a great deal more in other places (1,500 operations a year are carried out in Britain, 80,000 a year in North America). The alternative to surgery is small doses of aspirin and no need for a stay in hospital.

The problems for research in a 'two tier' health service do not stop with fragmentation itself but in the quite different requirements the government may place on those hospitals staying in and those opting out. The possibility of a double standard operating with respect to medical audit is a very real one — the government has not made it clear whether it will require opted-out hospitals to provide data on their performance.

Opportunities for good epidemiological work are also threatened by the possibility of the disappearance of the NHS Central Registry. As analysis has been able to replace mere description, epidemiology has provided clues both for prevention and intervention. The government's approach to what it sees as too many general practitioners, may lead to a system in which patients go directly to consultants, as they do in many other Western European countries, with primary care being given by secondary carers. The breakdown of the system in which patients'

The government says that it wishes to maintain a healthy environment for clinical research. That is a very welcome assertion, but nothing like enough information is being provided to show how it intends to do so. . . . At present the government's position remains untenable — the apparent failure to address the problems of research is completely at variance with its stated aim to improve the NHS.

notes follow the patients around will be a blow not only to continuity of care but also to research. Changed patterns of patient referral may have equally serious consequences for other parts of medicine. For effective clinical research, enough patients with particular diseases, or complex clinical conditions, must be concentrated in one institution. So there is little point in attempting research except in harness with the provision of a first-class clinical service. Patterns of referral to academic units in teaching hospitals which provide this level of service have often taken many years to establish. These patterns could be destroyed very easily by over-concern with costs of treatment in such centres, which will tend to be of the most modern, with knock-on costs in laboratory services. On the other hand, experience in the Netherlands, for example, suggests that patients may be sent to centres of excellence from peripheral hospitals, to spare local budgets.

Working Paper 2 of the White Paper states the government's intentions to introduce cross-charging of, on the one hand, the costs of clinical teaching and research to the NHS, and on the other, the provision of patient care by clinical academics and research workers. Understanding the real costs of research is likely to be beneficial. But badly carried out cross-charging will simply throw obstacles in the way of the effective running of the present three-way collaboration between medical schools, the NHS and the research financiers, whether charities or the Medical Research Council.

The final threat to research lies in the

possibility of changes in the pay and conditions of employment of NHS clinical staff. The problems of recruiting high-quality people into academic research will be worsened if differences in salaries between NHS and academic staff are further accentuated. Kenneth Clarke (Secretary of State for Health) does not foresee any great discrepancies there. Indeed, in his speech to the medical profession of 10 July 1989, he suggested that he would be prepared to approach the Department of Education and Science for supplements to clinical academics' pay. One can only hope that the Minister's assurances hold good.

Service costs

Again, in the same speech Kenneth Clarke announced a number of measures intended by the government to support medical education and medical research. Among them was the proposal to distribute an additional £5.5 million through Service Increment for Teaching (SIFT) to teaching hospitals, paid to provide for the service costs of research. This has been welcomed, although enthusiasm is tempered; not only is the sum very small, but the distribution of SIFT is based on undergraduate teaching numbers which do not actually correlate with research activity. Mr Clarke's intention to seek better research indicators in the future, and the intention for self-governing hospitals to be able to make additional payments to research workers undertaking NHS work (see above), are essentially positive proposals. It was also significant that he recognized that additional resources would be needed to support service costs of research in non-teaching hospitals.

Worries remain about the possibility that patterns of patient referrals might well be destroyed; about the future of specialist units within NHS hospitals; and about pay differentials. It is also disappointing that the government is not prepared to take a strong line on clinical research being an essential part of the work of hospitals that have opted out.

The government says that it wishes to maintain a healthy environment for clinical research. That is a very welcome assertion, but nothing like enough information is being provided to show how it intends to do so. There is clearly still a long way to go before those who believe in the improvement of health by research, and who put their money where their mouths are, will be convinced that the government believes the same and will act accordingly. At present the government's position remains untenable — the apparent failure to address the problems of research is completely at variance with its stated aim to improve the NHS. □

John Galloway is at the Cancer Research Campaign, 2 Carlton House Terrace, London SW1Y 5AR, UK.

Transgenic route runs into sand

A spectacular claim that spermatozoa can carry foreign DNA into mouse eggs to make transgenic mice has not been confirmed, but there are no grounds for saying that it should never have been published.

SPECTACULAR claims make the most cruel disappointments. That is one of the lessons to be learned from the appearance in the current issue of *Cell* of a report of several parallel failures to repeat what seemed, as recently as June this year, to be a simple way of generating transgenic mice — animals carrying genes from a different species or even genus. Another may be that most genomes are remarkably resistant to accidental pollution by foreign DNA.

The tale is nevertheless interesting in itself, and also important for its bearing on the management of the scientific literature. Earlier this year, an Italian group from Rome reported their success in making transgenic mice by a variation of a standard *in vitro* fertilization process in which foreign DNA was mixed with the spermatozoa used for fertilizing mouse eggs (Lavitrano, M. *et al.* *Cell* 57, 717; 1989). Put simply, the foreign DNA was supposed to be carried into the embryos by the spermatozoa, shown by the same investigation to have a propensity for picking up DNA. Nearly a third of the emergent mice were said to carry the foreign genes, which were said to be transmitted faithfully to a succeeding generation of offspring.

A report such as this was bound to engender both excitement and controversy. The excitement is easily understood. Transgenic mice are invaluable laboratory tools, chiefly as means of inferring how the expression of genes is regulated. There are now several techniques for making them, ranging from the micro-injection of DNA into fertilized egg cells to the use of retroviruses carrying segments of foreign DNA together with appropriate controlling elements. But whatever the technique, a transgenic mouse is necessarily a hit-or-miss construction. Among the reasons for the excitement over the Italian report last June is the simplicity of the technique and the success rate reported — nearly a third of the viable artificial embryos emerging seemed to carry foreign DNA.

The reasons why the original report must have aroused controversy are also not far to seek. For one thing, it is surprising that individual spermatozoa can pick up foreign DNA, either as circular plasmids or straight pieces of helical DNA, yet Lavitrano *et al.* reported that up to 4,000 copies of a plasmid (loaded with an anti-

biotic resistance gene for easy detection) may be taken up by a single sperm. Given that, it may have been less surprising that the foreign DNA seems to be integrated in the mouse genome, but last June's speculation that the mechanism might have been responsible, in the course of evolution, for the transfer of genetic material between species was bound to send shivers down many a spine. In a comment accompanying last June's publication in *Cell*, Max L. Birnstiel and Meinrad Busslinger understandably compared the report with the earlier reports of what is now known as 'cold fusion'.

That is the excitement and the controversy. The disappointment is the letter from four well-known mouse-genome manipulators (Ralph L. Brinster, Eric P. Sandgren and Richard R. Behringer, all from the University of Pennsylvania, together with Richard D. Palmiter from the University of Washington at Seattle) in the latest issue of *Cell*. They say that they have failed to confirm the integration of foreign DNA into artificially fertilized mice in eight series of experiments, two of which followed essentially the same experimental protocol as the Italian. For good measure, they also report that seven other laboratories, spread from Budapest to Pasadena, have similarly negative results.

So what can have gone wrong? One possible answer is simply, "Nothing". *Cell* also publishes in its most recent issue a letter from Lavitrano *et al.* that is a model of how people whose results are not confirmed should respond to challenge: "the inability to reproduce our results . . . suggests . . . a serious problem of which we were not aware", "when we received news of negative results . . . we re-examined the procedure . . . no obvious point has so far emerged", and so on. By the account of Brinster *et al.*, the authors have also readily responded to requests for reagents and other materials that would allow exact replication of their protocols. Lavitrano *et al.* volunteer that, while continuing to study their transgenic mice, they are also planning that their work should be repeated at another laboratory. It may yet emerge that the original report was correct, but valid only in the particular circumstances of the Rome experiment. It seems unlikely that the phenomenon described will be more generally valid.

Meanwhile, attention will concentrate

on one still-puzzling feature of the original report. The plasmid DNA apparently easily incorporated into mouse spermatozoa and thus into mouse genomes was distinguished by two sites at which it could be cut by a particular restriction (cutting) enzyme, but the foreign DNA incorporated into intact mice seemed to have lost one of these sites. Everybody concerned has followed the original suggestion that there must be some process at work that rearranges the DNA before it is incorporated, but that is almost as puzzling a circumstance as the possibility that spermatozoa can pick up thousands of copies of plasmid DNA from the medium that surrounds them.

So there remains the question provoked by Brinster *et al.*, who round off their report of their failure to replicate the original results with some homilies on the proprieties of the publication process. They make four points, three of which are platitudinous: exciting research reports do not always emerge from distinguished laboratories; the more "important" are claims, the more quickly will they be tested; and verifications (or the opposite) should first be reported in science journals, even if they are afterwards put out in press releases. Almost everybody will agree.

The fourth point is the puzzle. Brinster *et al.* say that "the central aspect of the process of verification is the objective and methodological resolution of a question . . . best accomplished in a cooperative manner . . . as is occurring . . . in this case." They add that, whatever the outcome of the investigations now planned, "those in research, and others . . ." will have learned a lot about "how scientific knowledge evolves" (*sic*).

Will they? Brinster *et al.* would have done better to acknowledge that there is a further question to be asked: is the peer-review system the universal safeguard against error it is supposed to be? In an ideal world, referees can keep on asking questions of authors who report extraordinary results until all possible doubts are exorcised, but that is not the real world in which we live. Implicit in what Brinster *et al.* say is their assumption that people with remarkable results to report have a right to see them published quickly. But may it not be that it is the extraordinary results that need most checking time?

John Maddox

EARTHQUAKES

New rumbles on deep sources

Cliff Frohlich

As ordinary materials become ductile at the pressures and temperatures that must exist 70–700 km beneath the Earth's surface, how is it possible for earthquakes to occur there? A persistent dialogue for the past 60 years between seismologists and experimental geophysicists has revealed much about the structure and mechanics of the Earth's mantle, but the origin of deep-focus earthquakes remains unidentified. On page 733 of this issue¹, Green and Burnley present new laboratory data that suggest a plausible solution to the problem.

The dialogue began in the 1920s, when Harold Jeffreys dismissed suggestions that some earthquakes occur at great depths by pointing out that the Earth's mantle had no strength. Laboratory experiments show that at high temperatures and pressures, rocks ordinarily do not fracture catastrophically (see figure), as they do in earthquakes. Instead, under applied stresses they deform in a ductile fashion, like pitch. Jeffreys knew that the Earth's mantle must flow in this way because we observe glacial rebound in response to the disappearance of great masses of ice, and because the Earth fails to support mountains with elevations greater than 10,000 metres or so. In 1928, however, Kiyoo Wadati² published a remarkably thorough study of the seismograms of some Japanese earthquakes, proving conclusively that some of them really do occur at depths of many hundreds of kilometres.

Thus was born the paradox of deep earthquakes. How can they possibly occur, in the light of Jeffreys's objections? This is an important question, especially as in a typical year about one quarter of all earthquakes recorded occur at depths exceeding 70 km. (The recent Californian earthquake, of course, does not belong to this category, having arisen at about 18 km depth.)

The first suggestion was that deep earthquakes might be implosions, occurring when olivine in the mantle suddenly transforms to a more dense spinel structure. Although the pressures necessary to transform natural olivine to spinel were not obtained in the laboratory until 1966, as early as 1936 J.D. Bernal had suggested that a phase transformation must occur. Bernal inferred this from laboratory experiments with olivine's germanate analogues, in which the silicon in $(\text{Mg,Fe})_2\text{SiO}_4$ olivine is replaced by germanium. This produces a synthetic rock that undergoes phase changes at a fraction of the pressures of natural olivine. But does a phase transformation produce deep earthquakes? The seismologists' clear and immediate response³ was that the

radiation patterns from deep earthquakes possess quadrants of compressional, as well as dilational, motion, unlike that to be expected from an implosion.

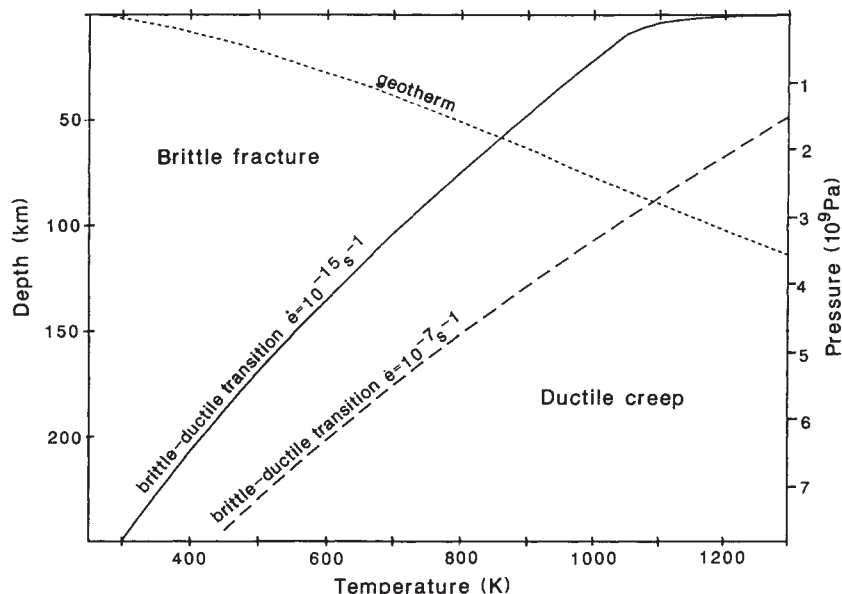
In short, deep earthquakes look much like shallow earthquakes. But, as suggested by the figure, they cannot be produced by the same mechanism. Moreover, theories of fracture have been successful at explaining shallow earthquakes as the result of the opening and coalescing of myriads of tiny cracks. Straightforward physical arguments suggest that it is physically impossible to open cracks at mantle pressures.

Occasionally, ideas are so attractive that they are maintained despite contrary evidence. So it has been with the idea that phase transitions are related to deep earthquakes. In the past half century, more than a thousand papers concerning deep earthquakes⁴, earthquake mechanics and mantle rheology have appeared. It is now certain that phase transformations occur in mantle materials at depths near 400 km (olivine–spinel) and 670 km (spinel–oxide), close to the depths of the deepest earthquakes. Plate tectonics provides a means of conveying cold rock, and possibly wet sediments, down past these depths in the deep earthquake zones. Surely phase changes in these materials are linked with the earthquake process?

Green and Burnley's suggestion is an interesting development. From low-pressure experiments^{1,5} on the olivine-analogue

germanate Mg_2GeO_4 , they find that the phase transition depends critically on the shear stress as well as on the hydrostatic stress. Although stress is relieved by ductile flow at both low and high temperatures, they find a narrow temperature range where there is unstable, catastrophic failure as tiny lenses of spinel form and link up as 'anticracks' along the plane of maximum shear stress. A shear-stress dependent mechanism depending on the occurrence of transitions along a planar region is attractive for several reasons⁶. It does not require that a large volume of material changes phase during a large earthquake. And it could explain why seismologists find no deep earthquakes beneath about 670 km, because at about this depth the spinel–oxide transitions are complete and no more phase transformations are available.

In short, deep earthquakes are fundamentally different from shallow earthquakes. Or are they? In 1966 Raleigh and Paterson⁷ suggested that the presence of water disassociating from serpentine rocks might change the effective stresses at the source of deep earthquakes, and even keep cracks open. Fluids injected in wells near Denver had apparently lowered the effective stress and caused a series of shallow earthquakes beginning in 1962. If such a mechanism explains the Denver earthquakes, why could it not work at greater depths as well? Expressed in terms of the figure, the effect of the fluid is to alter the mechanics so that everything behaves as if it were at shallower depths. Serpentine is basically just olivine and water, manufactured in oceanic lithosphere when ocean water interacts with crustal



Relationship between pressure (depth), temperature, strain rate ϵ and 'ordinary' rock failure. As either temperature or pressure increases, the rock failure process changes from a catastrophic fracture (earthquakes) to ductile flow. Because the geotherm meets the failure curve at pressures corresponding to depths of 70–100 km, we expect earthquakes to be confined to the Earth's lithosphere. Even if the temperature is hundreds of degrees cooler in subduction zones, ordinary brittle fracture cannot occur at the depths of the deepest earthquakes. What, then, is the mechanism that allows them to occur? (Figure from ref. 4.)

and mantle material. At sufficiently high temperatures and pressures, serpentine disassociates into olivine and water, and thus at greater depths this might allow cracks to open and earthquakes to occur.

This hypothesis seems to be substantiated by laboratory studies presented at a recent meeting of the American Geophysical Union by Charles Meade and Raymond Jeanloz⁹. They find that only when serpentine is present can they measure acoustic emissions (presumably caused by microscopic earthquakes) in tiny samples of olivine squeezed in their diamond-anvil apparatus and heated to mantle conditions. These results are preliminary, and one may object that even if water or another liquid is present, it seems unlikely it would achieve pore pressures sufficiently high to open cracks. But they will be important if they hold up, because they seem to demonstrate earthquake-like failures in samples of real mantle materials at pressures and temperatures found in the mantle.

In addition, Meade and Jeanloz⁹ find acoustic emissions in stressed samples of pure silicon and pure germanium at pressures corresponding to depths of 1,500 km in the mantle. Although no-one believes that pure silicon or germanium occur in the mantle, these experimental results are significant because they prove that phase transitions can produce earthquake-like behaviour at pressures well above the brittle-ductile transition. The troublesome wrinkle is that such behaviour apparently may be produced by more than one possible mechanism.

So what should we believe? Do deep

earthquakes occur because of phase transitions, because of fluids, or for some entirely different reason? Are deep earthquakes mechanically like shallow earthquakes, or fundamentally different? Fifty years of science has turned up several possible ways to resolve Jeffreys and Wadati's dilemma, of which Green and Burnley's is the latest. Currently, the biggest uncertainty with their results is that they perform their experiments on germanate analogues at only about one-tenth of the pressures at the focus of the deepest earthquakes. For their proposed mechanism, which acts only over a narrow range of temperature, it needs to be demonstrated that the analogy between germanate and silicate behaviour holds true under more severe conditions. The good news from the new work is that laboratory rock-mechanics experiments are finally approaching conditions in the mantle, and so the dialogue about the deep earthquake mechanics can be fuelled by laboratory observations instead of mere speculation. □

Cliff Frohlich is in the Institute for Geophysics, University of Texas, Austin, Texas 78759, USA.

1. Green, H.W. & Burnley, P.C. *Nature* **341**, 733–737 (1989).
2. Wadati, K. *Geophys. Mag.* **1**, 161–202 (1928).
3. Leith, A. & Sharpe, J.A. *J. Geol.* **44**, 877–917 (1936).
4. Frohlich, C. A. *Rev. Earth planet. Sci.* **17**, 227–254 (1989).
5. Burnley, P.C. & Green, H.W. *Nature* **338**, 753–756 (1989).
6. Kirby, S. J. *geophys. Res.* **92**, 13789–13800 (1987).
7. Raleigh, C.B. & Paterson, M. J. *geophys. Res.* **70**, 3965–3985 (1965).
8. Meade, C. & Jeanloz, R. *EOS* **69**, 490 (1988).
9. Meade, C. & Jeanloz, R. *Nature* **339**, 616–618 (1989).

PALAEONTOLOGY

Neck and neck

Michael A. Taylor

THE superb fossil reptiles from the Middle Triassic sediments of Monte San Giorgio in Switzerland¹ have attracted intense research interest for more than half a century, but there are still some intriguing problems to be solved. One of the more puzzling is the lifestyle of *Tanystropheus*, a reptile with a neck half the length of its entire body, yet made of only twelve vertebrae, each one grotesquely elongated. Despite the fine fossil evidence available, the function of this neck is not fully understood even after an extensive study by Wild^{2,3} and a new interpretation by Tschanz⁴.

The 230-million-year-old Monte San Giorgio sediments, now on top of a mountain in the Alps, were deposited in a small, nearly land-locked basin 10 km in diameter with access to the Tethys Sea. They have yielded a range of sizes of the largest *Tanystropheus* species, *T. longobardicus*, from small juveniles 50 cm long

to adults up to 6 m long. The adults were predators, catching fish and cephalopods with conical, recurved teeth, as confirmed by stomachs containing hooklets from cephalopods' arms. Nevertheless, they were poorly streamlined and do not seem to have been efficient swimmers. They used their webbed hind limbs for propulsion in the water, but were fully able to walk on land. The tail may also have been used for swimming but can hardly have been essential, as specimens have been found in which the tail has been shed by autotomy, like modern lizards, presumably to distract predators.

The most problematical aspect of *Tanystropheus* is its grossly elongated neck. Wild suggests^{2,3} that it was flexible and used for catching food: the animal would have used the head and neck for fishing for prey, while the rest of the body remained on the shore. Alternatively, the animal could have dabbled when floating

at the surface (like modern ducks) or darted at prey while swimming. When walking on land, the animal would have held up its neck in a sigmoid curve, rather like a swan.

But Tschanz⁴ argues that the neck was both too stiff and too weak for such a lifestyle. The thin, elongated ribs of the neck vertebrae ran below and to either side of the vertebral column, each overlapping several other vertebrae. These ribs stiffened the neck against vertical bending. The relatively shallow vertebrae provided very little area of attachment and very short lever arms for the neck muscles which were probably too weak to pull the neck up into an S-curve against gravity, especially a neck stiffened by ribs.

In Tschanz's view the animal had to hold its neck more or less straight out in front and would surely have found active life on land difficult: it may not even have been strong enough to have held its neck above the ground, let alone in a sigmoid curve. But in the water, the weight of the neck would have been relieved by buoyancy, and its stiffness would have helped the animal to swim without interference from bending of the neck and the resulting yawing.

The relatively long, unstreamlined body of *Tanystropheus* suggests that it either hunted slow prey or ambushed fast prey before it could escape⁵. This strategy would suit an animal living close inshore in relatively shallow water, where the bottom and plants provide cover. But the stiffened neck seems inappropriate for this strategy. Perhaps it could still dart its neck at prey, using the residual sideways flexibility to move its open mouth over the prey.

Juveniles of *T. longobardicus* had relatively short necks and teeth that were triply cusped like those of some insectivorous lizards. Wild suggests that they hunted insects and became fully marine only when adult. But Tschanz's view of the neck as a stiff structure applies to juveniles as well as adults, making it hard to see how juveniles could have caught insects at all. The teeth are similar to those of *Amblyrhynchus*, the modern marine iguana of the Galapagos, which feeds on algae, and one author has proposed that juvenile *T. longobardicus* did the same⁶. But the iguana has a short, flexible, powerful neck which helps it tear plant material from the rocks. It is hard to imagine a stiff-necked *Tanystropheus* doing this.

It may be that guessing the function of the long neck is the wrong approach to the problem. A comparison of growth in several species of *Tanystropheus* and related forms suggests that increases in the relative length of the neck preceded overall increases in body size in evolution^{2,3}. Wild concluded that the long neck was an adaptation for efficient foraging,

enabling the body size to increase.

But Tschanz's analysis, using different baselines, indicates that most of the growth of the neck of *T. longobardicus* during life can be explained as a strong and almost constant positive allometry: the neck length is linearly related to a power greater than unity of the animal's dimensions. Furthermore, a similar pattern of growth is found in the related but much smaller *Macrocnemus bassanii*. This needs confirmation from other species, but implies that the neck of *T. longobardicus* is as long as one would expect in any *Tanystropheus* of this size. Its great length in this species may therefore be an indirect consequence of the evolution of large size for some other reason, such as sexual competition or defence. Also, the positive allometry of

neck growth in *T. longobardicus* seems to be weaker than in *M. bassanii*, hinting that this, the largest species of *Tanystropheus*, was approaching the longest viable length of neck — hardly surprising as the animal risked tipping over onto its nose every time it came out onto land. So, perhaps, *T. longobardicus* had a long neck simply because it was a big animal, and it survived in spite of it rather than because of it. □

Michael A. Taylor is at the Leicestershire Museums, 96 New Walk, Leicester LE1 6TD, UK.

1. Bürgin, T., Rieppel, O., Sander, P.M. & Tschanz, K. *Scient. Amer.* **260** (6), 50–57 (1989).
2. Wild, R. *Schweiz. paläont. Abh.* **95**, 1–162 (1973).
3. Wild, R. *Mem. Soc. géol. France N. S.* **150**, 37–44 (1987).
4. Tschanz, K. *Palaeontology* **31**, 997–1011 (1988).
5. Massare, J. A. *Paleobiology* **14**, 187–205 (1988).
6. Cox, C. B. *Nature* **254**, 654–655 (1975).

SURFACE PHYSICS

Controlling crystal growth

Colin Humphreys

EPITAXY, from the Greek 'arranged upon', describes a mode of crystal growth in which the atoms of one crystal are registered perfectly on the atoms of another. (DNA can also be regarded as a kind of epitaxial structure, Watson and Crick originally suggesting that one DNA helix 'crystallizes' on another which acted as a template.) Epitaxial structures are becoming increasingly important in materials science, physics, chemistry and electronics. For example, solid-state lasers can be built from epitaxially grown layers of two different semiconductors, say GaAs and AlGaAs. For most man-made structures the aim of epitaxy is to grow crystal A upon crystal B with an atomically flat and defect-free interface between A and B. But if the deposited material A has a larger surface energy than the substrate B, A will roll up and form islands on B. Now, M. Copel *et al.* suggest (*Phys. Rev. Lett.* **63**, 632–635, 1989) a novel way to overcome this problem: the use of a surfactant, which is a 'wetting agent' that changes the surface energy so that A spreads out as a thin film on B. The idea may eventually give increased control over epitaxial growth and enable epitaxial structures that have previously been thought impossible to be grown.

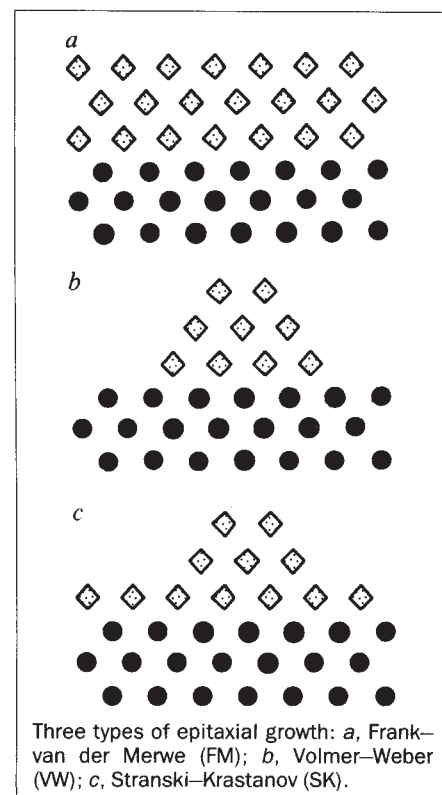
With real control of epitaxy, new materials could be fabricated atomic layer by atomic layer, each layer acting as a template for the next. This has yet to be achieved for alternating monatomic layers of different compounds, but the possibility that single crystals of such materials could have radically new properties makes the quest for perfect epitaxy a leading focus in materials science. The main impediment to this is that if A wets B, then B does not wet A, so that growing high

quality ABAB multilayers is difficult and the use of surfactants is potentially very important.

In practice, epitaxy occurs according to one of three distinct growth modes as shown in the figure: layer-by-layer (Frank–van der Merwe (FM) growth); three-dimensional island growth directly on the substrate (Volmer–Weber (VW)); and a mixture of the above — initially layer-by-layer growth for a few layers followed by island growth (Stranski–Krastanov (SK)). If A and B are identical materials then a single crystal can of course be grown, layer-by-layer, by various experimental techniques. But if A and B differ, their atomic spacings will differ and the result will be a strained epitaxial system. It can be shown theoretically that layer-by-layer growth of a strained system is not possible in equilibrium, which would seem to rule out the potential of epitaxial growth. Fortunately, kinetic constraints on mass transport can result in layer-by-layer growth and the resulting structures, although not in equilibrium, are metastable and can last for a long time.

As mentioned above, whether growth occurs layer-by-layer or via islands in such structures depends upon relative surface energies: if the deposited material A has larger surface energy than the substrate B, then island growth will tend to occur. (The situation is slightly more complicated than this because interface and strain energies must also be considered.) The problem is analogous to that of a liquid drop on a surface. Will it spread out to make a monolayer thin film (like oil on water) or will it form an island (like mercury on glass)?

Copel *et al.* have performed both



calculations and experiments on the epitaxy of germanium and silicon, of which Ge has the lower surface energy. Hence, Si grows on Ge by island growth (VW mode), but Ge grows on Si initially layer-by-layer, then by island growth (SK mode). Copel *et al.* show that a monolayer of arsenic can be used to reduce the surface energies of both Ge and Si. In addition, the As segregates to the surface during growth (there is little point in using a surfactant if it stays at the interface).

The authors claim that by covering the substrate surface with As, they promote the layer-by-layer growth of both Ge on Si and of Si on Ge. If substantiated, this result will be very important. But further work is needed to characterize the quality of the epitaxial growth, and the extent to which As is incorporated in the growing layers must be more thoroughly investigated. Nevertheless, the control of epitaxial growth using surfactants as suggested by Copel *et al.* provides a potentially important new avenue to 'evade equilibrium thermodynamics' and produce high-quality epitaxial layers of structures that have previously proved impossible. We know now that enzymes mediate the growth of one strand of DNA upon another. Could biology be pointing the way to growth of perfect man-made epitaxial structures using mediators such as surfactants? □

Colin Humphreys is in the Department of Materials Science and Engineering, Liverpool University, PO Box 147, Liverpool L69 3BX; from 1 January 1990 he will be in the Department of Materials Science and Metallurgy, Cambridge University, Cambridge CB2 3QZ, UK.

Twitching and switching

Allen Selverston

SINGLE-CELL recordings from both vertebrate and invertebrate neuronal networks suggest that certain types of neurons can be identified by their unique physiological characteristics. Usually, the local connections made by the cell are unknown, but it is generally assumed that each cell's functional connections are constant, as they are what give the cell its particular signature. That this may not be the case has recently been demonstrated in both the lobster¹ and the crab².

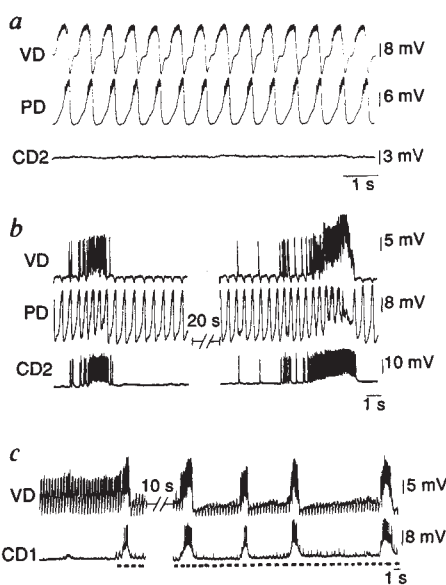
In the case of the lobster, Hooper and Moulins show that a neuron—the VD cell—that has long been considered part of the central pattern generator controlling the rhythmic movements of the posterior foregut, or pylorus, can instead switch to the network that controls the rhythms of the anterior foregut, or cardiac sac, whenever this network is active. The underlying mechanism for the switch can be ascertained because much of the synaptic connectivity is known³ and new information about sensory feedback and neuromodulation for the stomatogastric system has recently become available⁴⁻⁶.

The VD cell receives synaptic input both from other cells of the pyloric central pattern generator and from neurons of the cardiac sac network. When the cardiac sac rhythm is silent, the VD neuron bursts in time with the pyloric pattern. Like the other pyloric neurons, it has intrinsic bursting pacemaker potentials whose expression depends on descending inputs from higher centres to the stomatogastric ganglion. In addition, when active, the VD and the other pyloric cells express 'plateau' properties so that a short depolarizing pulse triggers a long-lasting depolarizing plateau. These active membrane properties plus the extensive synaptic connections act in concert to produce the normal pyloric pattern (part *a* of figure).

Either by stimulating a particular sensory receptor in the stomach wall or by activating the receptor's nerve directly, Hooper and Moulins were able reliably to activate the cardiac sac rhythm *in vitro*. Remarkably, this activation was accompanied by an instantaneous switching of the VD neuron from the pyloric pattern to the cardiac sac pattern (parts *b* and *c* of figure). How does this come about? The pyloric synaptic inputs to VD remain intact, as indicated by rhythmic synaptic potentials. But, surprisingly, all the regenerative properties of the VD neuron, bursting and plateau potentials, totally disappear.

With the active membrane responses in VD absent, the cell now responds to the strong input from the inferior ventricular

(IV) cell of the cardiac sac network. The other cells of the pyloric rhythm respond transiently to mechanoreceptor stimulation, but immediately return to their normal activity upon cessation of stimulation. This is strong evidence for the release of a neuromodulator during the stimulation of the mechanoreceptors and its exclusive action on the VD neurons, causing them to become passive but allowing them to be activated by bursts of synaptic excitation from the IV cell (part *b* of figure). It is this mechanism that appears to be the basis for the switch from one network to the other.



The VD neuron can fire either with *a*, the pyloric network (PD neuron trace) or *b*, the cardiac sac network (CD2 neuron trace). *c*, Tonic stimulation (dotted line) of a sensory nerve, the lpln, activates the cardiac sac network (CD1 neuron trace), and the VD neuron switches from the pyloric network pattern (left) to the cardiac sac network pattern (right). Taken from ref. 1.

Direct stimulation of the IV inputs does not suppress the regenerative properties of the VD cell, so the source of the putative neuromodulatory substance must originate elsewhere.

Previous work from Moulins's laboratory has shown that a single cell of an upstream ganglion releases acetylcholine into the stomatogastric ganglion, activating muscarinic receptors⁷. Such neuromodulators appear to act by activating receptors which trigger second messenger systems thereby altering both cellular properties⁸ and synaptic strengths while the modulator is present and for some time afterward. As a result, there can be a major functional 'rewiring' and significant alteration of the pyloric pattern. Subsequently, many laboratories have

demonstrated both the presence and the physiological effects of different modulators on both the pyloric and gastric mill of the stomatogastric system⁹. Amines and peptides are particularly effective; both can be delivered to the stomatogastric central pattern generators either via the blood or by direct local release into the neuropil of the stomatogastric ganglion¹⁰ (although there has been no evidence to suggest that they cause neurons to switch networks).

Sensory feedback loops, which can alter the pattern via conventional synaptic action¹¹, are a recently described source of neuromodulators. One feedback loop uses stretch receptors in both gastric and pyloric muscles (the gastro-pyloric receptors) to affect the gastric pattern on a cycle-by-cycle basis while modulating the pyloric pattern over longer periods¹². Remarkably, these receptors contain both acetylcholine, which acts on nicotinic receptors of gastric cells, and serotonin, which acts on particular cells of the pyloric system. An interesting possibility, therefore, is that the modulator affecting the VD cell is released by the sensory neurons which are stimulated to activate the cardiac sac rhythm.

The fact that VD switching occurs following mechanical stimulation in semi-intact preparations argues that this phenomenon may be of biological significance in lobsters, but it is not yet clear how the loss of the VD neuron affects the functioning of the pyloric system. As VD is a motor neuron, the muscle it controls must perform a different role when it is part of the cardiac sac rhythm. But the precise nature of the behavioural effects for each separate rhythm remain to be elucidated.

That the phenomenon described by Hooper and Moulins may be widespread is suggested by the report at a recent conference² of comparable findings in the crab foregut. Dickinson, Mecsas and Marder reported that the red-pigment-

1. Hooper, S.L. & Moulins, M. *Science* **244**, 1587-1589 (1989).
2. Dickinson, P.S. in *Proc. 2nd int. Congress Neuroethology: Neural Mechanisms of Behavior*, Berlin, 10-16 September 1989 (eds Erber, J. et al.) 184 (Thieme, New York, 1989).
3. Selverston, A.I. & Moulins, M. *The Crustacean Stomatogastric System* (Springer, New York, 1987).
4. Simmers, J. & Moulins, M. *J. Neurophysiol.* **59**, 757-777 (1988).
5. Katz, P.S. & Harris-Warrick, R.M. *Neurosci. Abstr.* **13**, 821 (1987).
6. Marder, E. et al. *New Insights into Synaptic Function* (eds Edelman, G.M., Gall, W.E. & Cowan, M.W.) 305-327 (Wiley, New York, 1987).
7. Dickinson, P.S. & Nagy, F. *J. exp. Biol.* **105**, 59-82 (1983).
8. Katz, P.S. & Harris-Warrick, R.M. *J. Neurophysiol.* **62**, 558-570 (1989).
9. Harris-Warrick, R.M. *Neural Control of Rhythmic Movements in Vertebrates* (eds Cohen, A.H., Rossignol, S. & Grillner, S.) 285-332 (Wiley, New York, 1988).
10. Turrigiano, G. & Selverston, A.I. *J. Neurosci.* **9**, 2486-2501 (1989).
11. Simmers, J. *The Crustacean Stomatogastric System* (eds Selverston, A.I. & Moulins, M.) 242-250 (Springer, New York, 1987).
12. Katz, P.S. & Harris-Warrick, R.M. *J. Neurophysiol.* **62**, 571-581 (1989).

concentrating hormone increases the synaptic interactions between the cardiac sac and gastric mill neurons to such an extent that the entire gastric mill fires with a cardiac sac rhythm. Virtually all of the gastric neurons must be switching to allow the operationally independent gastric mill and cardiac sac central pattern generators to act as one functional unit.

The idea of sharing neurons between networks may confer some as yet unexplainable computational advantage on small systems. It adds further complexity

to a specific well-defined system already shown to be extremely versatile in its response to modulators. Moreover, it suggests caution in interpreting the data from single cells of more complex systems; if the chemical environment can switch a neuron from one circuit to another, the hypercomplex cell of one preparation may be the simple cell of another. □

Allen Selverston is in the Department of Biology, University of California, San Diego, La Jolla, California 92093, USA.

METEORITICS

Antarctic differences of opinion

I. P. Wright and Monica M. Grady

THE flux of interplanetary debris delivers meteorites more or less uniformly to all parts of the Earth's surface, yet there is evidence to suggest that the meteorites collected in Antarctica represent a different population from that found elsewhere. Such evidence was presented at a recent meeting* to discuss this distinction, and there was much debate as to whether any differences were real or artefacts. Those with partisan views were unlikely to have changed their opinions about the meaning of the apparent differences between Antarctic and non-Antarctic meteorites in the light of presentations at the meeting. But the idea that the two sample sets may represent some sort of difference in meteorite sources remains a fascinating possibility. The effects of terrestrial weathering must first be constrained, however, if the differences are to be attributed to pre-terrestrial phenomena.

The first evidence of a distinction was presented by M. E. Lipschutz and colleagues (J. E. Dennison *et al.* *Nature* **319**, 390–393; 1986) on the basis of volatile/mobile trace-element compositions of H5 ordinary chondrites collected from Antarctica and elsewhere. Another apparent difference reported since is that certain meteorite types have thus far only been found in Antarctica. These include polymict brecciated eucrites, lunar meteorites and low FeO ureilites (the last of which also have distinctive oxygen isotope compositions). Antarctic and non-Antarctic carbonaceous chondrites show differences in chemistry, oxygen isotope composition, extent of aqueous alteration and heating; these effects are undoubtedly due to preterrestrial processes. But do the observed differences document separate meteorite populations or do the data simply extend the currently known ranges in the properties of individual meteorite groups?

Another difference between Antarctic and non-Antarctic meteorites is the higher

ratio of H- to L-group chondrites in the Antarctic collection. On closer examination, however, the enhanced ratio is the result of an excess of H-group chondrites from just one Antarctic collection area. Thus, the recent addition of a single shower fall of H-group chondrites to this region would distort the H/L ratio of Antarctic meteorites, which would otherwise be similar to that from the rest of the world (G. Huss, University of Chicago). Critics of this hypothesis include R. Harvey (University of Pittsburgh), who warned that the shower would have had to be anomalous to explain the relatively large numbers of small fragments present in the Antarctic collection. The continued recovery of meteorites from Antarctica should help to settle this debate.

If the distinctions are real, what are the reasons? Are the differences the result of preterrestrial processes, so that they can yield interesting clues about the Solar System, as Lipschutz (Purdue University) maintains? Or is a mixture of selection and weathering effects at work? An important distinction between Antarctic and non-Antarctic meteorites is the range of their terrestrial ages: samples collected from observed falls are no more than about 200 years old; by contrast, Antarctic finds may have fallen over the past million years. It is not expected, however, that the parent population has changed over this period. The temporal decay of a meteorite flux, attributable to collisions in the asteroid belt, takes 10^7 years (G. W. Wetherill *Nature* **319**, 357–358; 1986).

Also, the mean size of Antarctic meteorites is much less than that of non-Antarctic samples. Clearly, this is because small fragments are more readily seen when exposed on ice rather than elsewhere. But frequently, numerous small fragments originate from a single fall that broke up in the atmosphere, on impact or subsequently. Thus, although collections include more Antarctic meteorites than non-Antarctic, statistical analyses should rely on their total mass, and not their

number, to be reliable.

Complications arise from loss mechanisms that must operate in the Antarctic, the effects of which are not fully understood: small fragments are blown away by the wind, and large fragments may sink into the ice. Furthermore, the collected mass for non-Antarctic meteorites can be much less than that which originally struck the atmosphere. For example, the Revelstoke fireball caused huge atmospheric detonations and the impact was recorded by seismographs, yet the collected meteorite amounted to only 1 gram. Similarly, immediately following a meteor shower over India, only a few large pieces were collected. Over the following three years, however, a coordinated search with the assistance of school children expanded the collection by several hundred pieces.

The idea that the differences are pre-terrestrial is not supported by the cosmic-ray exposure ages of H-group chondrites (L. Schultz, Max-Planck-Institut für Chemie), by their major- and minor-element chemistries (E. Jarosewich, Smithsonian Institution), or by their indigenous carbon content and isotope composition (M. M. G.), none of which vary between Antarctic and non-Antarctic samples. If the meteorites are from different sources, then the first finding would imply that two independent source bodies broke up by chance at the same time to supply the putative separate populations.

A possible explanation of the chemical and isotope differences that do exist is terrestrial weathering. Unfortunately, the nature of terrestrial weathering is not well understood: M. A. Velbel (Michigan State University) demonstrated the ineffectiveness of the weathering categories used by NASA's Antarctic meteorite curatorial facility. The modest improvement in weathering classification (to indicate the presence of surficial evaporite deposits) seems a long way short of an adequate description of the weathering histories of Antarctic meteorites. It is difficult to conceive a convenient and rapid screening procedure (over 1,000 meteorite fragments were collected in the US collection programmes in the 1988–89 season alone). Furthermore, the effects of weathering might extend to considerable depth within a meteorite sample (D. W. Mittlefehldt, NASA Johnson Space Center, Houston). Researchers who analyse 'interior' samples should be aware that such materials are often obtained by simply breaking open a meteorite — it is probable that fracturing takes place along pre-existing cracks, which may have been inundated with terrestrial fluids. □

I. P. Wright and Monica M. Grady are in the Department of Earth Sciences, The Open University, Walton Hall, Milton Keynes MK7 6AA, UK.

* Differences Between Antarctic and Non-Antarctic Meteorites, University of Vienna, 27–28 July 1989.

Convergence of drug action

Robert B. Freedman

Two papers on pages 755 and 758 of this issue^{1,2} add a further intriguing twist to the emerging connection between immunosuppressant drugs, the cytosolic proteins that bind them and the enzymatic activity of those proteins. Earlier this year, Fischer *et al.*³ and Takahashi *et al.*⁴ showed that the protein, cyclophilin, which binds the immunosuppressant drug cyclosporin A with high affinity and is assumed to mediate its action, has prolyl-peptidyl *cis-trans* isomerase (PPIase) activity. Their work also showed that cyclophilin is identical to a previously identified PPIase⁵, and that the PPIase activity is specifically inhibited by cyclosporin. Now, two groups that have been exploring the action of the structurally distinct immunosuppressant, FK506, report^{1,2} that it, too, is bound by a specific cytosolic binding protein with PPIase activity, but that this binding protein is quite distinct from cyclophilin.

The two groups, Siekierka *et al.*¹, at Merck, Sharp and Dohme, and Harding *et al.*², at Harvard and Yale Universities, were following up the finding (see ref. 6) that the macrolide FK506 is a distinctly more potent immunosuppressant than the cyclic peptide, cyclosporin, which is widely used for prevention of graft rejection in organ transplantation. Siekierka *et al.*, having previously shown that FK506 bound to a cytosolic protein⁷, purified this protein from the JURKAT T-cell line. Harding *et al.* have synthesized affinity matrices containing cyclosporin- and FK506-derived ligands for affinity chromatographic purification of the corresponding binding proteins from human and bovine tissues. Both groups found that the purified FK506-binding protein is distinct from cyclophilin in terms of size and N-terminal sequence (and, indeed, is unrelated to any sequence in the current database). Both groups used radioligand binding assays and competitive binding studies to characterize the ligand binding activity; Siekierka and colleagues found that FK506 binds to its protein with a 1:1 stoichiometry, and an affinity close to 1 nanomolar.

Most importantly, both groups demonstrate that the purified FK506-binding protein has PPIase activity, although the activity towards the conventional PPIase substrate (a substituted proline-containing tetrapeptide) is 20–25-fold lower than that of cyclophilin. And finally the groups show that cyclophilin and the FK506-binding protein are quite distinct in terms of specificity; cyclophilin binds, and is inhibited by cyclosporin and shows no recognition of FK506, whereas the converse holds for the FK506-binding protein. This difference in specificity is seen most clearly

in the kinetic results of Siekierka *et al.*, which show complete inhibition of each receptor by its corresponding ligand at concentrations where the other drug has no effect whatsoever.

Minor differences between the results reported in the two papers indicate that the story is far from complete. The binding protein purified by Siekierka *et al.* has a relative molecular mass (M_r) of 11,000 and shows saturable ligand binding at concentrations in the nanomolar range, whereas the protein purified by Harding *et al.* has a M_r of 14,000 and is not saturated at ligand concentrations up to 1 micromolar. Although these discrepancies may derive from the fact that the proteins come from different species, and from the use of distinct FK506 derivatives as radiolabelled ligands, possibly a diverse family of proteins is involved. Siekierka and colleagues do not report any sequence for their protein, so it is not certain that the proteins purified by the two groups are identical.

In the light of earlier data, which

indicate that FK506 and cyclosporin act on T-lymphocytes in essentially equivalent fashion⁸, the new data suggest that the two drugs act through distinct pathways but that their modes of action converge on PPIases. Current data show that these proteins are abundant and are important in mediating the activation of T cells, but how and why this activation exploits their ability, as PPIases, to catalyse rotamerization of peptide bonds to prolyl residues, remains completely mysterious. Far less mysterious is the fact that this new clue to the action of a class of drugs with multi-million-dollar sales, is now generating vigorous research activity. □

Professor Robert B. Freedman is in the Biological Laboratory, University of Kent, Canterbury, Kent GT2 7NJ, UK.

1. Siekierka, J.J., Hung, S.H.Y., Poe, M., Lin, C.S. & Sigal, N.H. *Nature* **341**, 755–757 (1989).
2. Harding, M.W., Galat, A., Uehling, D.E. & Schreiber, S.L. *Nature* **341**, 758–760 (1989).
3. Fischer, G., Wittmann-Liebold, B., Lang, K., Kiefhaber, T. & Schmid, F.X. *Nature* **337**, 476–478 (1989).
4. Takahashi, N., Hayano, T. & Suzuki, M. *Nature* **337**, 473–475 (1989).
5. Lang, K., Schmid, F. X. & Fischer, G. *Nature* **329**, 268–270 (1987).
6. Thomson, A.W. *Immunology Today* **10**, 6–9 (1989).
7. Siekierka, J.J., Staruch, M., Hung, S.H.Y. & Sigal, N.H. *J. Immun.* (in the press).
8. Tocci, M. J. *et al. J. Immun.* (in the press).

ASTRONOMY

Galactic chemical evolution

R. F. Carswell

DISTANT galaxies, that is those at high redshift, are seen as they were at a much earlier epoch in the history of the Universe. From their forms and distributions, it is hoped to learn what unseen effects in earlier epochs led to their formation. From their spectra, it is hoped to learn their chemical compositions which, in turn, should tell us about the progress of stellar nucleosynthesis in the early Universe. Two groups now report^{1,2} the measurements of the spectral lines of zinc in galaxies illuminated by bright background quasars. Because zinc is one of the few elements that do not become bound into interstellar grains (where they would be invisible), these spectra give us the best measure yet of early nucleosynthesis.

Element synthesis in the primordial fireball during the first few thousand seconds of the Universe yielded almost exclusively isotopes of hydrogen and helium. All the other elements, referred to as the heavy elements, were made later in stars after they and galaxies coalesced out of the initial material distribution. The heavy-element enrichment of the interstellar medium in our Galaxy and in others occurs when the processed materials from stars is ejected, either relatively slowly through stellar winds or explosively if the star becomes a supernova. This medium

partakes in subsequent star formation, nuclear burning and further enrichment of the interstellar medium.

Information on the details of the chemical history of our Galaxy has been obtained from studies of the heavy-element abundances in the atmospheres of stars for which age estimates are available. The overall picture is that some initial enrichment of elements such as carbon, nitrogen and oxygen occurs relatively quickly from processing in short-lived, high-mass stars. After 10^9 years or so the iron-group elements appear in the interstellar medium when longer-lived, lower-mass stars yield significant quantities of material. The rate of enrichment depends on the relative numbers of stars at various masses and their formation rate, the fraction of the stellar ejecta which escapes from the Galaxy completely, and any accretion of primordial material into the Galaxy.

An independent study of the chemical history of galaxies can be made by analysing the element abundances at high redshifts when they (and the Universe) were much younger. At present, the only way of doing this with any reliability is to examine the spectra of bright high-redshift quasars for absorption lines from hydrogen and heavy elements which arise

in gas in the galaxies that lie between us and the quasar. The strengths of these lines may be used to infer both the relative gas-phase abundances of the ions responsible, and, through some corrections for relative populations of different levels of ionization, the relative element abundances in the interstellar gas.

There is a major complication that has to be considered if one wishes to infer the true abundances from the gas-phase abundances. In the interstellar medium in the Galaxy, much of the material for the common elements (such as carbon, oxygen, silicon and iron) goes into the formation of interstellar dust grains, so that the true abundances of these elements are somewhat higher than the gas abundance would indicate. Only for a few relatively rare elements, notably zinc, is there negligible depletion onto dust, and only through measurement of the abundances of these are we likely to obtain an accurate picture of the heavy-element abundances in high-redshift systems.

Detecting the zinc lines is difficult because of the weakness of the lines even for abundances typical of those in our Galaxy, and the possibility of lower abundances at high redshift increases this difficulty. Despite this, two groups have recently reported measurements of lines from singly ionized zinc which allow them to determine its abundance relative to hydrogen at redshifts $z = 2.8$ and $z = 2.3$ (refs 1, 2). Thus, depending on the rate of slowdown of the expansion of the Universe, they are examining the chemical content

of material which existed when the Universe was something like 15–30 per cent of its present age. In both cases, the heavy-element abundances relative to hydrogen, as measured by Zn^+ , are roughly an order of magnitude down on the value near the Sun. The straightforward interpretation of these results is that nucleosynthesis in these high-redshift systems has led to the production of only about one-tenth of the heavy elements we see today, and so the objects are in an early stage in their chemical evolution.

In both studies it was possible to compare the zinc abundance with chromium, another species that is depleted onto grains, and in both cases the chromium abundance is closer to the true value than in the Galaxy. As a consequence, it seems likely that these high-redshift regions are relatively dust free. If this is true in general, abundance analyses based on the stronger lines of more common elements that are depleted in a galaxy should provide reasonably reliable estimates for the true abundances. This is encouraging for those wishing to probe higher-redshift, and possibly lower-abundance, systems to obtain a fuller picture of the chemical history of material in the Universe. □

R.F. Carswell is at the Institute of Astronomy, Madingley Road, Cambridge, CB3 0HA, UK.

1. Meyer, D.M., Welty, D.E. & York, D.G. *Astrophys. J.* **343**, L37–L40 (1989).
2. Pettini, M., Bokkenberg, A. & Hunstead, R.W. In *The Epoch of Galaxy Formation* (eds Frenk, C.S. et al.) 107–112 (Kluwer, Dordrecht, 1989).

MULTIPLE SCLEROSIS

Hopeful genes and immunology

Dale E. McFarlin and Peter J. Lachmann

ALTHOUGH both environmental and genetic factors are believed to contribute to the pathogenesis and etiology of multiple sclerosis (MS), it is the genetic contribution which is attracting most attention in research laboratories at present, and which was the main focus of a recent workshop*.

The evidence for a genetic contribution to MS is derived from studies of the disease in families and ethnic groups. Recent population-based studies of large numbers of patients have confirmed earlier impressions that up to 18 per cent have affected family members (A.D. Sadovnick, University of British Columbia) and that there is a higher concordance in monozygotic (MZ) than in dizygotic twins (G.C. Ebers, University of Western Ontario). Also, some clinically normal MZ twins have abnormalities on magnetic resonance imaging consistent with subclinical disease. Moreover, environmental factors

do not explain why the disease is absent in Hutterites residing in western Canada, a region of high prevalence (W.J. Hader, University Hospital, Saskatchewan) or why in Kuwait, the prevalence is approximately three times greater in Palestinians than in Kuwaitis (A.S. Al-Din, Kuwait University).

The epidemiology of MS is consistent with a polygenic disorder and a number of genes were discussed as possibly contributing to susceptibility. Emphasis was placed on genes related to immune function because of the prevailing opinion that an immunopathologic process gives rise to the disease. Associations between MS and HLA class II molecules, particularly the DRw2 and DQw1 serotypes, are well known. Extensive analyses of HLA class II genes in MS have been conducted and a *DQB1* gene that encodes polymorphic sequences in DR2, DR4 and DRw6 was found in 97 per cent of Norwegian patients and in 70 per cent of controls by using a sequence-specific oligonucleotide probe

(F. Vartdal, University of Oslo). Another new finding was that a recently recognized DQ α polymorphism correlates with the disease (R. Heard, Hammersmith Hospital). So far, however, no specific HLA class II sequence related to susceptibility or resistance to MS, analogous to that described in insulin-dependent diabetes, has been identified.

The possible role of genes other than those of the *HLA-D* locus on chromosome 6 was discussed, particularly those encoding TNF- α , TNF- β and complement components. Some products of these genes can lead to damaged myelin and secretion of TNF- β by myelin basic protein-specific T-cell clones, discussed below, correlates with the production of experimental allergic encephalomyelitis (EAE), a possible animal model of MS (L. Steinman, Stanford University). Complement activation has been implicated in the pathogenesis of MS and EAE (D. A. S. Compston, University of Cambridge), and it was suggested that genetic deficiency of late-acting complement components could be protective in populations with low frequency of MS.

Three independent groups of investigators presented new findings linking MS to T-cell receptor (TcR) genes. One study showed that haplotypes for germline V beta genes in DR2⁺ MS patients differed ($P < 0.002$) from those in DR2⁺ controls (W.E. Biddison, NIH). In the second study, *TcR β* haplotypes defined by polymorphic markers were compared in MS sibling pairs, and haplotype sharing was found to be significantly more frequent ($P < 0.004$) than expected (S.L. Hauser, Harvard University). The third study reported linkage of MS to a *TcR α* gene polymorphism and described amplification of this gene by polymerase chain reaction (PCR) from MS brain (J.R. Oksenberg, Stanford University).

The influence of genes at other loci was also considered. The previously described association between MS and genes on chromosome 14 including *GMI* and the α -antitrypsin gene were debated and now seem less convincing to some investigators (G. Stewart, University of Sydney; B. De Hooghe, University Hospital, London, Ontario). No new information on the previously reported association with *GM3* was described. Various clinical observations provoked discussion of genes related to gender: the prevalence is higher in females than males; in like-sex MZ twins, the concordance is higher in females than males, and in families in which parent to child 'transmission' occurs, the father to son 'transmission' is lower than other possibilities (Sadovnick). A resistance gene on the Y chromosome or a recessive resistance gene on the X chromosome could explain the findings, but data supporting such possibilities are lacking.

New findings from a variety of auto-

* *Genes and Susceptibility to Multiple Sclerosis*, Cambridge, 25–29 July 1989.

immune animal diseases were presented, from whence a common theme with implications for MS emerged. This is that the spontaneous occurrence of autoimmune disorders, such as thyroiditis, insulin-dependent diabetes and systemic lupus erythematosus — and so, by implication MS — requires the interaction of three or more genes, one of which is usually an *MHC* gene. When fewer than the required number of genes are present, pathological abnormalities may occur in the absence of clinical disease. For example, at least three genes are required for the expression of insulin-dependent diabetes in NOD mice, but if only two are present as in NOD.H2^b congenic strain, abnormal accumulations of mononuclear cells occur in the pancreas (L. Wicker, Merck, Sharp and Dohme). Multiple genetic influences are clearly operative in experimentally induced demyelinating diseases that serve as possible models for MS, including EAE (F. Lublin, Thomas Jefferson University, Philadelphia) and encephalitis associated with coronavirus in rats and Theiler's murine encephalomyelitis virus (TMEV) in mice. A gene that confers resistance to subacute demyelinating disease in rats has been identified (H. Wege, Universität Würzburg). Recent experiments indicate that the demyelination produced by TMEV is the consequence of a T-cell response to a viral antigen (S.D. Miller, Northwestern University). Such immunologically mediated processes require recognition of antigen in association with MHC molecules, but in the central nervous system, MHC is expressed in relatively low amounts, if at all. This has led to considerable interest in the capacity of residual cells of the central nervous system, such as astrocytes, microglia and capillary endothelial cells, to function as antigen-presenting cells and to participate in immunopathological processes.

The findings in MS and animal models provide a clear rationale for future studies. One approach will be to define the disease susceptibility genes related to MS in greater detail. Such efforts will benefit from recently developed approaches such as 'micro-satellite' probes and the use of PCR in conjunction with specific oligonucleotide primers for the genes of interest. Assessment of multiple genes in well-characterized families will be essential. This will be laborious and progress will be enhanced by cooperation among different investigators. It was particularly encouraging, therefore, that considerable discussion, including an unscheduled evening session, was devoted to future collaborative efforts. As such genetic studies proceed, there will be parallel efforts to characterize immune reactivity in MS. Extensive studies in a few patients indicate that such findings will be complicated. For example, T-cell reactivity to myelin basic protein is

heterogeneous both in terms of epitope specificity and *TcR* gene usage (J.R. Richert, Georgetown University).

Another line of investigation that received encouragement is the development of therapeutic strategies that involve immunological manipulation. Studies of EAE and immune reactivity to myelin basic protein provided the basis for five approaches affecting either T cells or antigen presentation. One is treatment with antibody to CD4. This inactivates the subset of T cells that bear this protein (H. Waldmann, University of Cambridge) and is beneficial in EAE. Another approach is the use of antibody to the *TcR*. Support for this approach is derived from experiments with encephalitogenic T-cell clones. In PL mice, these preferentially use V β 8.2, and the administration of antibody to this *TcR* gene product blocks EAE (Steinman). An alternative is the use of antibody to MHC class II molecules, which suppresses EAE in some species.

Finally, there are two approaches that make use of peptides and which were explored recently in News and Views by Charles Janeway (*Nature* 341, 482–483; 1989). In one case, the plan is to immunize with *TcR* peptides. Recent data indicate that encephalitogenic T cells from mice and rats share germline *TcR* genes despite difference in peptide specificity. A conserved sequence has been identified and vaccination with a corresponding synthetic nonapeptide induces resistance to EAE in the highly sensitive strain of Lewis rats (M. Howell, Immune Response Corporation, San Diego). The other possibility is to block MHC class II molecules with specific peptides. Assessment of synthetic polypeptides with single amino-acid substitutions in the nonapeptide that causes EAE in PL mice has identified one that binds with high affinity to MHC class II molecules (J. Rothbard, ImmuLogic Pharmaceutical Corporation, Palo Alto) and inhibits the induction of EAE (D. Wrath, Stanford University).

Collectively, these experiments provide an elegant demonstration that recently acquired knowledge in fundamental immunology can be used to modify an immunologically mediated disease such as EAE. This produced considerable excitement for extending such efforts to MS patients. Implicit in such reasoning is that a T cell-mediated process contributes to the pathogenesis of MS and that humans will respond in the same manner as rodents. Neither of these is yet certain. Nonetheless, it is clear that the workshop has provided the basis of a variety of new approaches for studying MS. □

Dale E. McFarlin is in the Neuroimmunology Branch, National Institute of Neurological Disorders and Stroke, Bethesda, Maryland 20892, USA; Peter J. Lachmann is in the MRC Molecular Immunopathology Unit, University Clinical School, Cambridge CB2 2QH, UK.

Sound of silence

A PORTABLE radio or tape player with loudspeakers is essentially an offensive weapon; its chief function is to annoy other people. The socially responsible music lover uses a personal tape player driving earphones, so as to deafen himself without disturbing anyone else. Sadly, this noble intention is seldom achieved. The soft, tinny, rhythmic leakage from earphones has a unique annoyance value of its own. So Daedalus is developing a truly private listening system. His idea is to vibrate the user's eardrums directly, without ever generating any sound at all.

To couple the eardrum directly to a mechanical transducer would require the most delicate surgery, and would permanently affect normal hearing. But Daedalus recalls a magnetic ear-lacquer he once invented. Sprayed into the ears, it coats the eardrums so that they can be vibrated by an oscillating magnetic field. It was intended as the sensing component of a magnetic metal-detection system; but Daedalus now sees it as the ideal way to create an entirely private sound sensation. With his eardrums coated with ferromagnetic lacquer, the user will directly hear the audio field of a nearby induction coil, mounted perhaps on his collar or spectacle frame. Feed the coil from his personal tape player, and he will hear its music in wonderful high fidelity — all the distortions and limitations of small earphones will be eliminated. Nobody else will hear a thing. The lacquer should last for several days, until displaced by the ear's natural waxy secretion.

This splendid technology can easily be extended. The deaf-aid industry will surely welcome such a simple way of generating subjective sound, free of all the limitations of small ear pieces and the problems of acoustic feedback. Buses and aircraft will be able to radiate a music channel magnetically, and charge patrons for the use of a discreet ear-spray to listen to it. And magnetic tape recordings will become far more accessible — just wind them past your lacquered ear and hear them directly, with no electronics needed at all!

But the uses of ferromagnetic ear-lacquer should extend far beyond sound reproduction technology. Electricians could listen directly to motors, transformers and relays to diagnose their ills. They could track wiring through a house, and estimate the current flowing in it by sound alone. And health worriers could monitor the general environmental level of a.c. magnetic fields, which over time are feared to depress the human immune system. With the malign tones of overhead power lines, military radars, and badly shielded video-display terminals ringing in their ears, they could assess the hazards directly, and adopt the appropriate level of worry.

David Jones

Satellite data contamination

SIR—Strong¹ claims to have observed a global warming of $0.1\text{ }^{\circ}\text{C yr}^{-1}$ for the period 1982–88 using satellite-derived sea surface temperatures (SSTs). His results show a much larger warming trend than other analyses of the same data². Strong's analysis is in error because, during the initial two years of his analysis period, he excluded SST data when the satellite data were contaminated by the signal from the dust cloud from the El Chichón volcanic eruptions of 29 March and 3–4 April 1982. Because the dust from the El Chichón volcanic cloud absorbed some of the infrared radiation emitted by the surface and re-emitted some infrared radiation at a lower temperature, satellite measurements of the thermal emission of the sea surface gave readings in these areas that were too low.

The excluded data in this region were not randomly distributed with respect to the mean SST, but covered the region of high SST anomalies resulting from the great El Niño of 1982–83. It was precisely where record-breaking SSTs were being observed at the surface that Strong eliminated data. In fact, recognition of the intensity of the 1982–83 El Niño was delayed at the time by dependence on satellite data that did not show the warm SSTs.

Strong^{3,4} used the difference between the satellite-derived SST and *in situ* data from ships and buoys as a measure of the thickness and location of the El Chichón dust cloud. It can be seen from his analyses that the *in situ* SST measurements in the region of the El Chichón dust were more than $0.4\text{ }^{\circ}\text{C}$ higher than the satellite-retrieved values between the Equator and 30° N from April 1982 to August 1983, and were more than $1.0\text{ }^{\circ}\text{C}$ warmer for most of this region for most of this time.

The result of excluding the contaminated satellite-derived data was to miss the warm temperatures of the ocean during the first two years of his analysis. Thus the trend in his analysis is much too large, because it starts from temperatures that are too cold for 1982–83. Omitting the data from these two years did not correct this mistake, because the temperatures for these two years were higher than the average for the next two years, not equal.

Had Strong substituted *in situ* data for the missing data in 1982–83 to get a 'blended' analysis, as is done routinely at the Climate Analysis Center⁵, he would have calculated the correct trend. Indeed, he had these data available because he used them in his previous work^{3,4}.

Analyses of the long-term temperature record of the globe show an irregular warming during the past century (as

shown in ref. 2 for example), but this warming cannot be unambiguously ascribed only to the effects of anthropogenic greenhouse gases. To separate out the competing effects of volcanic eruptions (which cause cooling for several years, and probably longer), tropospheric aerosols, and random, chaotic variations of the climate system, the actual record of climate change must be known as accurately as possible. This requires a combination of data sources, including satellite data. But when satellite data are not available, such as in 1982–83, the best available data must be used to give correct global temperatures.

ALAN ROBOCK

Department of Meteorology,
University of Maryland,
College Park,
Maryland 20742, USA

STRONG REPLIES—As mentioned by Robock, the effects of aerosol from El Chichón during the 1982–83 period have been well documented^{5,6}, showing extensive involvement for about 18 months over large portions of primarily the Northern Hemisphere. I also discussed the effects from El Chichón. Robock is concerned that missing grid-point data (not eliminated by me, but by the Multi Channel Sea Surface Temperature (MCSST) cloud detection procedures) in the areas where aerosol contamination was extensive could distort the data, particularly if those areas were undergoing dramatic warming from the 1982–1983 El Niño. Although many grid boxes in the 10° – 30° N latitudinal band over the Pacific were void of data, most of these were during the period April–September 1982 before the development of El Niño during September 1982 along the Equator. By the time the volcanic debris migrated southward during December 1982 over the region being affected by the developing El Niño, some data were available for grid-box MCSST monthly means, although derived from fewer MCSST retrievals, despite volcanic aerosols. The missing MCSST data from the volcanic cloud, therefore, did not substantially affect the region where El Niño was developing.

The major concern is a period of negative offsets in derived MCSSTs from December 1982 to mid-1983 over the eastern and central equatorial Pacific. As Robock points out, an alternative analysis could have been performed to eliminate any possibility of contaminated MCSST data during the 1982–83 period by using a hybrid data set substituting entirely *in situ* sea surface temperatures (SSTs) everywhere for the El Chichón years.

Following Robock's suggestion, I have used monthly ship and buoy *in situ* SSTs

over a grid identical to the one I used earlier¹. These conventional data (not analyses) were then substituted for the initial 1982–1983 portion of the MCSST record. The revised short-term trends are somewhat less, but are still upward. For the 6.5-year record (January 1982–June 1988) the rates of change are: Global = $0.08\text{ }^{\circ}\text{C yr}^{-1}$; Northern Hemisphere (NH) = $0.07\text{ }^{\circ}\text{C yr}^{-1}$; Southern Hemisphere (SH) = $0.07\text{ }^{\circ}\text{C}$. This compares with my previously reported global, NH and SH MCSST rates of change of: 0.12, 0.16 and $0.10\text{ }^{\circ}\text{C yr}^{-1}$, respectively. Using entirely conventional data these global, NH and SH rates are: 0.04, 0.04 and $0.03\text{ }^{\circ}\text{C yr}^{-1}$. For hybrid data spanning an updated period from 1982 to March 1989 the global, NH and SH slopes are: 0.05, 0.04, and $0.05\text{ }^{\circ}\text{C yr}^{-1}$, respectively; conventional global, NH and SH SST rates: 0.03, 0.04, and $0.02\text{ }^{\circ}\text{C yr}^{-1}$, respectively. As well as accounting better for some of the contaminated MCSST data in 1982–1983, these updated values reflect the cooling that took place over much of the Earth's oceans during 1988. So far during 1989 the tendency has been again towards higher values.

ALAN E. STRONG*

Satellite Research Laboratory,
National Oceanic and Atmospheric
Administration,
Camp Springs,
Maryland 20233, USA

1. Strong, A.E. *Nature*, **338**, 642–645 (1989).
2. Reynolds, R.W., Folland, C.K. & Parker, D.E. *Nature* **341**, 729–731 (1989).
3. Strong, A.E., *Geophys. Int.* **23**, 129–141 (1984).
4. Strong, A.E. *Ocean–Air Interactions* **1**, 11–28 (1986).
5. Reynolds, R.W. *J. Clim.* **1**, 75–86 (1988).
6. McCormick, M.P., Swissler, T.J., Fuller, W.H., Hunt, W.H. & Osborn, M.T. *Geophys. Int.* **23**, 187–222 (1984).

*Present address: Oceanography Department, U.S. Naval Academy, Annapolis, Maryland 21402, USA.

High table tales

SIR—Robert May (*Nature* **341**, 386–387; 1989) has Haldane's famous beetle story elicited by a question from Jowett. Haldane was born (in Oxford) on 5 November 1892, Jowett died on 1 October 1893, having left Oxford in September of that year. So while they could have met, they certainly did not have a conversation either at high table at Balliol, or anywhere else.

MARK WILLIAMSON

Department of Biology,
University of York,
York YO1 5DD, UK

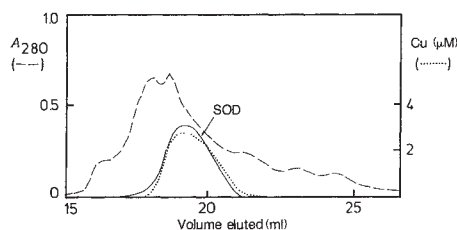
MAY REPLIES—Mundane constraints of time and space do not apply to stories about Oxford.

ROBERT M. MAY

Department of Zoology,
University of Oxford
Oxford OX1 3PS, UK

Mummified enzymes

SIR—Although it is known that many biopolymers in mummified tissues remain structurally intact, no functional evidence of an isolated protein has yet been found. The availability of a well defined air-dried mummy of a male aged 16 (± 2) years, from 1200 BC¹ prompted us to search for the presence of functional Cu₂Zn₂ superoxide dismutase or its remnants. A sample



Fast protein liquid chromatography of mummified brain extract on Superose-12. The column was calibrated with BSA, horse heart cytochrome c and glucagon, and was operated at 0.5 ml min⁻¹ at 20 °C; PBS was used as equilibration buffer. Protein elution was traced at 280 nm (A_{280}). The fractions were analysed for copper on a Perkin-Elmer Zeeman 3030 atomic absorption spectrometer. Superoxide dismutase activity was monitored using the nitro blue tetrazolium assay². The reaction volume (0.5 ml) contained 0.62 mM nitro blue tetrazolium, 20 mM Tris-HCl buffer, pH 7.8, 1 mM EDTA, 0.2 % (w/v) gelatine, 50 μ M xanthine, 0.18 μ M xanthine oxidase and the sample. Readings were taken at 540 nm in a 10 mm light-path cell at 23 °C. SOD, relative superoxide dismutase activity.

of brain tissue taken at autopsy and stored completely dry in the presence of solid NaOH, was minced and suspended in phosphate-balanced saline buffer overnight. Using the xanthine, xanthine-oxidase-dependent nitro blue tetrazolium assay², we obtained unequivocal proof of a specific Cu₂Zn₂ superoxide dismutase activity in the extract; 66 per cent of the copper present in the soluble fraction could be assigned to this enzymatic activity. The presence of 1 mM EDTA did not affect this activity, but 0.1 mM cyanide completely abolished the observed inhibitory reaction. Unfortunately, the concentration of the active component was too low to be detected by nitro blue tetrazolium staining on polyacrylamide gels after electrophoresis, but fast protein liquid chromatography of the extract on Superose-12 revealed a Cu-containing polypeptide of relative molecular mass 5,000 (5kD) to which we ascribed all the activity (see figure). It is assumed that during the ageing process, considerable portions of the original 31.3kD homodimer were

cleaved leaving the active core.

To the best of our knowledge, this is the first case of an enzymatically active component surviving 3,000 years of mummification. Secondary microbial infections after excavation and autopsy were excluded as a source of the superoxide dismutase activity, as no fungal or bacterial population was seen even when tissue extracts were incubated for more than 16 days. Other methods of mummification, specifically those using natron, resins, oils and especially bitumen, cause deterioration of many biopolymers. Muscle tissue samples taken from several mummies pretreated in this manner

showed considerably less activity, or none at all, probably because reactions of the resin or bitumen with intact tissue proteins, converted them into catalytically inactive polymers.

U. WESER
R. MIESEL
H.-J. HARTMANN

*Anorganische Biochemie,
Physiologisch-Chemisches Institut des
Universität Tübingen,
Hoppe-Seyler-Strasse 4,
7400 Tübingen, FRG*

W. HEIZMANN
*Hygiene Institut des Universität Tübingen,
Silberstrasse 7, 7400 Tübingen, FRG*

Screening for cystic fibrosis

SIR—P.N. Goodfellow, commenting on the probable identification of both the cystic fibrosis (CF) locus and the most common of the recessive alleles leading to the clinical condition, advocated an immediate start to carrier screening¹. Before prenatal screening can be implemented, I suggest that several issues have to be considered.

The defined mutant allele seems to account for about two-thirds of CF chromosomes, and hence is found in the homozygous state in about four-ninths of affected individuals²; these proportions may well vary in different populations. In the UK and North American populations, the frequency of affected individuals is around 1 in 2,000 so that about one affected pregnancy in every 4,500 of the screened population could be detected. The most likely strategy would be to screen one partner in each pregnancy; if he or she is a carrier, the second partner would be screened; and if both partners are carriers, prenatal diagnosis would be offered, with a view to termination of affected pregnancies.

In round terms, for first pregnancies the risk of CF would currently progress from the initial 1 in 2,000 (of which 1 in 4,500 are detectable), through 1 in 90 (1 in 140 detectable) for those screened and found heterozygous, to 1 in 4 where both partners are heterozygous (1 in 1,100 screened couples), with a residual risk of 1 in 250 where only one partner is finally found to be heterozygous for the known mutation (1 in 35 of screened couples). (The risks of not detecting an affected child are higher if whole families, rather than just the first child, are considered.) These figures will obviously be improved as further mutations are identified.

To enable this progression, informed consent to testing would be required from both partners; if screening were carried out on the first partner but consent was refused by the second, a positive test

would raise the risk to a level that might produce anxiety but would not necessarily justify prenatal sampling procedures. False-negative tests (unexpected affected children), as well as unnecessary prenatal sampling, would result from non-paternity of putative fathers; the frequency of non-paternity is variable, but can be 10 per cent or higher. The timing of the tests is difficult if screening is initiated at the first hospital ante-natal clinic (around 9–14 weeks gestation in Britain), because little time is left for obtaining the second and third samples if a reasonably early termination of pregnancy is to be offered. Earlier sampling, which implies the involvement of general practitioners, would be preferable.

Prospective parents would need education and counselling to give informed consent. Education should also include the relevant medical and para-medical specialities so that accurate and timely information could be provided. Community health workers would be needed to ensure appropriate timing, the co-ordination of tests with availability of facilities and continuity of counselling. High-risk couples should be referred to clinical genetics services experienced in ensuring that appropriate counselling and social support are available; and the outcome of the programme should be regularly reviewed.

Programmes of community health education and action have been instituted for ethnic groups with high incidences of haemoglobinopathies. Coping with genetic disease in this way would be a new and major undertaking for most of the European and North American population, and the uptake is hard to predict; but screening could not be justified without this kind of integrated approach.

ALISTAIR D. STEWART

*Yorkshire Regional DNA Laboratory,
Department of Chemical Pathology,
University of Leeds,
Leeds LS2 9JT, UK.*

1. Millet, N.B., Hart, G.D., Reyman, T.A., Zimmerman, M.R. & Lewin, P.K. in *Mummies, Disease and Ancient Cultures* (eds Cockburn, A. & Cockburn, E.) 71–84 (Cambridge University Press, 1983).

2. Younes, M. & Weser, U. *FEBS Lett.* **61**, 209–212 (1976).

1. Goodfellow, P.N. *Nature* **341**, 102–103 (1989).
2. Kerem, B.-S. et al. *Science* **245**, 1073–1080 (1989).

In the political orbit

John Noble Wilford

Journey Into Space: The First Thirty Years of Space Exploration. By Bruce Murray. W.W. Norton: 1989. Pp 381. \$19.95. To be published in Britain in February, £14.95.

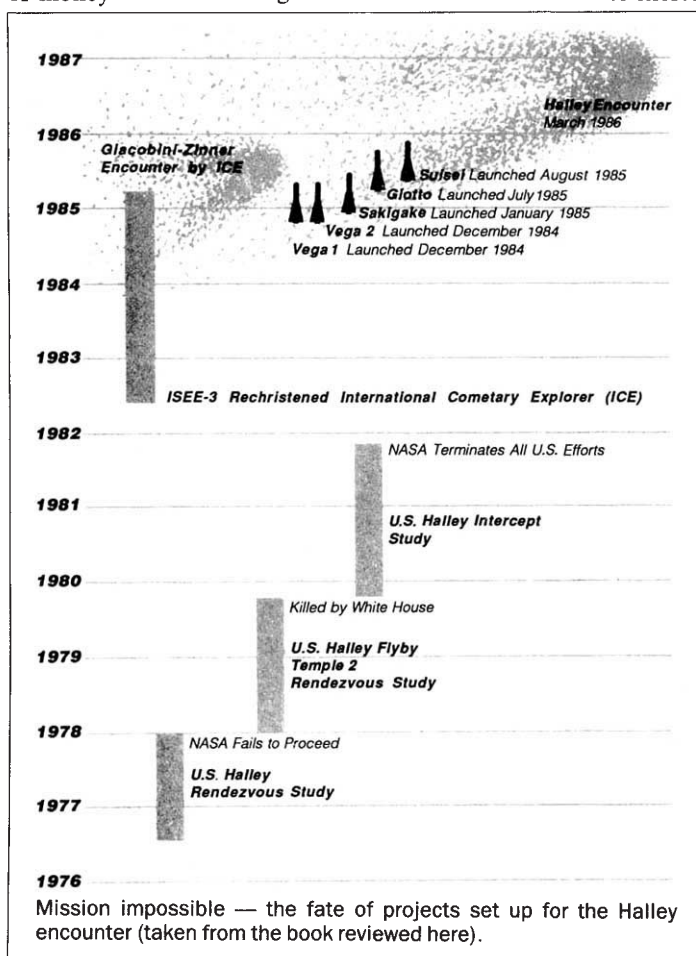
THE recent flight past Neptune by *Voyager 2*, the culmination of a 12-year journey of discovery in the outer Solar System, was a triumph for the brand of space exploration championed by Bruce Murray in his book *Journey into Space*. With a relatively modest expenditure of money compared to annual spending on the space shuttles, and at no risk to human life, the remotely controlled *Voyager* not only succeeded in visiting its primary targets, Jupiter and Saturn, but also managed to make it all the way to Uranus and, finally, Neptune. This wide-ranging reconnaissance of the planets ranks among the most exciting and scientifically rewarding achievements of the space age.

Murray has written a highly personal book. As a professor of planetary science at the California Institute of Technology, and a former director of the Jet Propulsion Laboratory (JPL), he has known the exhilaration of being in at the beginning of planetary exploration by space-craft. "What a time to be alive!" he exclaims (p.96) in recalling the heady days of preparation for the Mariner 10 mission to Mercury in the early 1970s. Commitments made in the previous decade were now bearing fruit. Hardly a year passed without the lift-off of another planet-bound craft or the arrival of more spectacular pictures from worlds that hitherto had been little more than smears of light in a telescope. Murray captures the excitement among scientists who look back fondly on those days as the golden age of planetary exploration.

Murray has also written an important and disturbing book. He takes us behind the scenes for glimpses of the conflict that has long split the American space programme into warring groups. The conflict has variously been characterized as manned versus unmanned flight, the engineers and bureaucrats of NASA versus scientists, or the powerful Houston and Huntsville space centres versus JPL, NASA's primary centre for unmanned planetary projects. Murray (who was on the losing side in most of the battles) presents a bloodied combatant's

view of events.

For those who deplore the dearth of new planetary missions over the past decade, which could have been worthy successors to the durable *Voyager* launched in 1977, Murray's tale of frustration in dealings with the White House and



NASA's own leadership should make them relieved that at least things were no worse. For those who wonder why there has been no rational, long-range US space policy since the Apollo Project, here are some of the answers. Murray does not pretend to be impartial, concluding a lengthy account of one struggle, he says that "political mediocrity triumphed over technical competence and dedication" (p.237).

As the director of JPL from 1976 to 1982, Murray found himself in the thick of the political wrangling over Galileo, an advanced spacecraft to orbit Jupiter and investigate its atmosphere, and over the various proposals for the United States to have its own entry in the international

flotilla being readied for the return of Halley's comet. This was for him an ultimately dispiriting task. He came to realize he had been "leading what proved to be a strategic retreat" (p.284) from the lofty ramparts previously occupied by planetary science.

Writing the book seems to have been a cathartic experience for Murray. He is asking his scientific colleagues to understand that he tried his very best, against all odds. With the administration of Jimmy Carter, he was working with a president who, more than any other, appreciated the scientific accomplishments, but failed to exert leadership in space policy or grasp

the symbolic value of space in global politics. Murray confides that in 1977 he had been asked to head NASA in the new Carter administration. Now he is left wondering if he could have made a difference. To take the job, he writes, would have cost him his scientific career, "leaving myself doomed to a purgatory of administration for the rest of my working life . . . A high price for four years of frustration" (p.169).

After his failure to win approval for a Halley's mission from the Carter White House, Murray made one final desperate attempt with the incoming administration of Ronald Reagan. In doing so, he confesses that he stooped to a blatant patriotic appeal. How could the United States, the leader of the free world, not be a part of the main Solar System enterprise of the decade? He was rebuffed again and instead discovered that some of Reagan's advisers, notably David Stockman, director of the Office of Management and Budget, were out to abolish the entire planetary programme. Murray recalls a call in early 1981 from a budget official, who asked: "What if J.P.L. were to disappear?" (p.268).

The rest of 1981, as Murray tells it, was a critical time for the US planetary programme. Not only did he have to fight off attempts to kill Galileo, which was originally scheduled for launch in 1982, but he also had to resist Stockman's moves to pull the plug, as it were, on *Voyager's* life-support system. If Stockman had had his way, there would have been no money for tracking *Voyager 2* beyond Saturn and thus no pictures from Uranus or Neptune.

Another adversary emerged at this time. Hans Mark, a former official in NASA and the Air Force, became deputy administrator at NASA with a hard-nosed agenda that included, the conversion of

JPL into a military research centre. In a meeting with Murray's bosses at Caltech, Mark "upbraided me" for putting the laboratory "in grave danger by obsessively pursuing planetary missions, delaying too long the inevitable move into defense work" (p.281).

A sadder but presumably wiser Murray writes of his eventual loss of innocence. Coming out of yet another meeting with unsympathetic NASA officials, he was struck with the insight: "NASA's constituency is the aerospace industry! Just what my cynical university friends have been saying for years" (p.335). Scarred by these assaults, he sometimes seems to yield to the temptation to use the book to even old scores. But the real 'heavy' of Murray's story is not Stockman or Mark. It is the space shuttle.

Development of the shuttle, beginning in 1972, marked another victory for NASA's dominant group, the proponents of expensive engineering programmes for manned flight. Like many scientists, Murray had opposed the shuttle-only policy all along. Astronauts only increased costs and mission complexity, and with its diminished post-Apollo budgets the shuttle left NASA with virtually no money for anything else. Murray seldom misses a chance to take a swipe at NASA's policy. "How", he asks, "did we drift from the Apollo reality to the Shuttle fantasy and, finally, to catastrophe?" (p.24). The answer comes in his comprehensive recounting of the political machinations as NASA struggled to defend its crumbling fortress, sacrificing nearly everything for the shuttle programme, and left to fight its battles without presidential support. No one at NASA, Murray tells us, was even allowed to question the shuttle-only policy — until it was too late.

At the outset, Murray asks a second question: "How can we once again rise to greatness in space?" (p.24). His proposed answer, which he raises in the final chapters, amounts to the offer of a truce between the proponents of manned and unmanned space flight. Murray suggests the new goal for the American programme should be Mars, to be explored in greater depth by further unmanned probes and eventually by astronauts. The two factions could thus work together towards a common destination. Murray outlines the current thinking of those who advocate that Mars should be the next target, and argues for a more serious consideration of Soviet proposals that the two nations should go to Mars together. These are indeed arguments that have received too little attention from US policy makers as they seek to establish a political justification for long-range goals in space exploration. □

John Noble Wilford is in the Science News Department, New York Times, 229 West 43rd Street, New York, New York 10036, USA.

On manus and pes

Bernard Wood

Functional Morphology of the Evolving Hand and Foot. By O. J. Lewis. Clarendon: 1989. Pp.359. £60, \$125.

COMPARATIVE anatomy is an unusual and difficult subject, and one that is far from finished. Its practitioners require flair, but most of all they need to be experienced, observant and painstaking. These attributes are not held in great esteem at present. Comparative anatomy is at best often regarded as antediluvian, science in a Victorian frock coat.

In this demanding and iconoclastic book, Lewis nails his colours unequivocally to the mast. He claims that as long as we continue to depend upon the fossil record for our understanding of evolutionary history, the interpretation of that record must be based on sound comparative anatomical knowledge. At the small risk of misrepresenting him, I believe that the author is suggesting that there has been too much 'soft' and too little 'hard' comparative anatomy. The 'soft' version consists of unsystematically consulting a few papers, old enough to be respectable, and extracting from their summaries factual items that support the particular thesis being put forward. The 'hard' variety requires both sufficient expertise to detect slipshod earlier work and the time and energy to supplement the good-quality information that does exist with one's own observations. It also involves developing a deeper comparative perspective and recognizing that the solution to a morphological conundrum among the primates might need to be sought among the muscles, tendons and bones of the monotremes.

The book is demanding because Lewis makes few concessions to his readers. The writing is clear, but the content is tough, much like the sinews it describes. There is little, if any, attempt to develop a consensus or seek a compromise, for, the author argues, there is a correct interpretation of form and that must be rigorously sought. Among the flesh and blood icons broken along the way are the practitioners of 'soft' comparative anatomy along with those who believe that it is enough to compare form through measurements and multivariate statistical analysis. Lewis's dissatisfaction with, if not disdain for, these tools is proper when they are used to compare specimens across too wide a range of morphology, but they do have a role when palaeontologists try to resolve more 'local' taxonomic problems. At first reading, there is the strong impression that the icons either all reside west of Bantry

Bay or have been publishing since 1960 — it was surely no mistake on the part of the author that a paper published two decades ago is described as a 'recent' review. This impression would, nonetheless, be an unfair slur on both the author and my North American colleagues; some of us in Britain receive just as vigorous a strapping as they do. Moreover Lewis is sufficiently even-handed to admit to one or two of his own morphological indiscretions.

The book is divided into two parts, almost equal in length, one on the hand and the other on the foot. The form of bones, joints and muscles are considered in turn. A final section of each part gives an account of the relevant primate and hominid fossil evidence, but in the light of a comparative perspective that ranges as wide as prototherian mammals and extends as far back as the Triassic.

There are weaknesses, but not many of them. I would have liked to have seen more reference to good-quality behavioural studies to support functional correlations, and photographs of whole animals in typical foot and hand postures would have laid even greater emphasis on functional morphology. Contemporary research in developmental biology may also render some of the arguments about evolutionary mechanisms obsolete before long. Heterochrony and its genetic basis are treated clearly, but a little naively. But the book's strengths are manifold, and stem from the author's sheer professionalism. Not only are Lewis's own studies meticulous, but they are clearly illustrated by his own drawings. The reading is wide and the conclusions are refreshingly direct. This volume will become a classic text on how we should regard the anatomy of the organs which, along with the brain, make us human beings.

Lewis's analysis of the manus and pes is unlikely to be anatomically faultless, but his interpretations are backed up with such a weight of evidence that they must all be taken seriously. If they are to be challenged and overturned, it must be on the basis of comparative evidence of similar quality — but are there practitioners with the experience, the understanding, the meticulous dissecting technique and the diligence to do it? □

Bernard Wood is in the Department of Human Anatomy and Cell Biology, University of Liverpool, PO Box 147, Liverpool L69 3BX, UK.

New in paperback

- *From Paradox to Reality* by Fritz Rohrlich. Published by Cambridge University Press, £9.95, \$14.95. See review in *Nature* 332, 189 (1988).
- *Psychosemantics: The Problem of Meaning in the Philosophy of Mind* by Jerry A. Fodor. Published by MIT Press, \$9.95, £8.95. Reviewed in *Nature* 331, 219 (1988).
- *Neurophilosophy* by Patricia Smith Churchland. Published by MIT Press, \$15.95, £14.25. See review in *Nature* 322, 413 (1986).

Yorick's luck

Mark Ridley

Sex and Death in Protozoa: The History of an Obsession. By Graham Bell. Cambridge University Press: 1989. Pp. 199. £25, \$44.50.

FOR half a century, from about 1880 to 1930, many biologists were busily trying to maintain perpetual, immortal cultures of protozoans. All the leading scientific nations contributed to this great research effort: it began with Germans, such as Engelmann and Weismann, was continued in France by Maupas and Balbiani, and the torch was passed, for the final phase, to such industrious Americans as Calkins, Jennings and Woodruff.

The main, though by no means the only, research organism was *Paramecium*. The work concentrated on 'isolate' cultures: no one doubted that 'mass' cultures with millions of protozoans could be kept going practically indefinitely. In an isolate culture, an individual protozoan is isolated and used to seed a new culture; from this culture, after a day or so, another individual is isolated and used in turn to seed a second culture, and so on. Some biologists claimed these cultures could be immortal; others that they inevitably senesced, as the organisms produced abnormalities and their fission rate declined.

There was another question. Could a senescent culture, if conditions were altered to permit sexual conjugation, be 'rejuvenated'? Again, some said yes, others no. And, according to Graham Bell, "the problem was never resolved; after fifty years it was simply abandoned". The body — or corpse — of that work has long since been buried under the dust of scholarship.

Bell's new book is a piece of grave-robbing, for he has read this old literature and has brought it to bear on our modern interests in the evolution of senescence, repair mechanisms, recombination and, particularly, sex. Incidentally, he was able to solve the old problem itself. He has located 75 experiments that can be analysed statistically. Of these, 65 show a negative relation of fission rate and time: the results (almost unknown to the experimenters) overwhelmingly confirm that isolate protozoan cultures do indeed 'senesce'. Without sex, a protozoan lineage will become extinct after a few hundred generations.

Bell also finds that sexual conjugation does revive a senescent lineage. The best experiment was by Calkins, who isolated conjugating couples from an otherwise asexual lineage of *Uroleptus mobilis*. Lines bred from the conjugants consistently out-lived the original parental lines.

Bell calls this "one of the more important experimental results of biology". It is certainly one of the more agreeable. Protozoans (in which, it should be recalled, sex is separate from reproduction) carry out conjugation in order to prolong their lives.

A number of other provocative results are conjured out of these old papers. But Bell's main concern is with the significance of the two principal results. In a chapter on senescence, he argues that decay of protozoan cultures is fundamentally different from senescence in multicellular organisms. He accepts the theory of Medawar and G.C. Williams, according to which animals like ourselves senesce because selection favours increased early survival at the expense of survival later in life. Bell believes that senescence in protozoan cultures is caused instead by the accumulation of deleterious mutations.

Reproduction in isolate cultures is parthenogenetic, by binary fission, and the accumulation of mutations thus illustrates one of the main theories of sex — the one known as 'Muller's Ratchet'. Sex, if this theory is right, exists to purge the DNA of the deleterious mutations that tend to build up in an asexual lineage. In Bell's neat image, sex is an exogenous repair mechanism. It is impossible, in a population of asexual organisms, to generate an organism with less than the present minimum number of deleterious mutations. This minimum number, moreover, tends to increase through time. If all the organisms with the current minimum number of mutations by chance all die without issue, the new minimum number

will increase. Muller's Ratchet will have turned. In a sexual population, an organism with fewer deleterious mutations can be generated by recombination, and the number of mutations therefore does not tend to increase. According to Bell, "the history of isolate cultures or protozoans provides as comprehensive an illustration of the Ratchet as one could wish for".

He is probably correct, but his interpretation is not so well established as he concludes. Other theories of sex could account for the same observations. A popular theory at present suggests that sex exists because of the coevolutionary relations of parasites and hosts. It is perfectly possible that, in an isolate culture, parasites to which that particular protozoan lineage lacks resistance genes will build up. Outcrossing could then introduce the resistance mechanisms, and rejuvenate the culture. Other phenomena, such as the spindly taxonomic distribution of asexual reproduction, which Bell explains by the Ratchet, can also be explained by most other theories of sex.

These are minor criticisms. This old and forgotten literature has turned out, in Bell's hands, to contain some unexpectedly suggestive results for more modern and fundamental problems. He has evidently enjoyed exhuming the 'musty volumes'. His book is concise, elegant, non-technical and (that rare thing) a thoroughly good read. □

Mark Ridley is in the Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 3EJ, UK.



The Eagle Nebula (M16) — a detail taken from one of the prints in *Precision Astrophotography*, a set of 10 (11 x 14 inch) photographs by Kim Zussman. The collection, together with two others in colour (*The Finest Galaxies* and *Deep-sky Photography*), is published by Kalmbach, 21027 Crossroads Circle, PO Box 1612, Waukesha, Wisconsin 53187, USA. Price is \$16.95 per set.

Starting with stars

John Lattanzio

The Fundamentals of Stellar Astrophysics.

By George W. Collins II. W.H. Freeman: 1989. Pp.494. \$47.95, £37.95.

THE theory of stellar astrophysics is naturally divided into the study of interiors and atmospheres. Collins has provided a convenient introduction to both subjects in the one text, aimed at first-year graduate students or advanced undergraduates. The book contains a thorough index, and provides a list of references and sources for further reading that will act as an excellent point of entry to the more advanced literature.

The text is divided into two parts, the first of which deals with stellar interiors. Unfortunately, the opening chapter contains a great deal of statistical mechanics. Although this material is clearly essential to the development of the subject, it may seem an anti-climax to the student impatient to deal with stars and an appendix would have been a better place for it. Collins gives appropriate attention to scaling arguments to familiarize students with the techniques, and his selection of theorems from Chandrasekhar's *An Introduction to the Study of Stellar Structure* is excellent. I feel that the section on nuclear energy generation is too brief, though it would serve as an introduction to the definitive treatment to be found in Clayton's *Principles of Stellar Evolution and Nucleosynthesis*.

Unexpected inclusions are the chapters on relativistic stellar structure and the structure of distorted stars. To my mind, however, the former would have been better omitted in favour of more detail in other sections. The final chapter of this part, which deals with pulsation and oscillation, seems to have been introduced for completeness. Ideally the stellar-evolution chapter would have mentioned variable stars (where they are seen in the Hertzsprung–Russell diagram, and how their evolution puts them there), and this chapter would then have been the necessity that it is.

Moving on to stellar atmospheres, Collins derives the equations of radiative transfer and illustrates various approximations which enable their solution. He then discusses the interaction between the gas and the radiation field. The chapter that should then be the reward for these toils, which is entitled "Construction of a Model Stellar Atmosphere", is somewhat disappointing because no example is provided.

The next two chapters deal with the formation and shape of spectral lines.

Collins then looks at departures from local thermodynamic equilibrium, and concludes with an account of complications which exist in real stars (stellar winds, for example) but which have been ignored in the standard theory.

More diagrams would have been appropriate (the account of stellar evolution, for example, contains but two HR diagrams), as would some example models of stellar interior and atmospheres both as illustrations of the concepts described in the text and to show the student the rewards of learning the physics and doing the calculations. Problems are given at the end of each chapter, and they cover a wide variety of styles. Some require a

stellar interior code or an atmospheres code for their completion! It is a pity that there is no section on the growing field of stellar seismology — future students of stellar astrophysics will be expected to be conversant with the subject.

These are minor criticisms of an otherwise thoroughly good book. An instructor could choose material from the text to highlight certain areas, and as a whole it forms a sound beginner's course for those wishing to specialize in stellar astrophysics. □

John Lattanzio is in the Institute of Geophysics and Planetary Physics, Lawrence Livermore National Laboratory, PO Box 808, Livermore, California 94550, USA.

Out of this world

Michael Rowan-Robinson

Encyclopedia of Astronomy and Astrophysics.

Edited by Robert A. Meyers and Steven N. Shore. Academic: 1989. Pp.807. \$49.95, £31.50.

THE reader turns to an encyclopaedia when he or she is looking for detailed information about an unfamiliar topic. These days science is so specialized, and its practitioners are forced to be so narrow in their professional interests, that even in a scientist's own field a high proportion of topics will come into that category. So there is every reason why astronomers (and others) need a book such as the *Encyclopedia of Astronomy and Astrophysics*.

There are two conditions that an encyclopaedia has to satisfy. The first is that the entries must be satisfyingly packed with useful facts and information; the second is that the encyclopaedia must give in total a reasonably comprehensive survey of the area being considered. Many of the entries in the present offering satisfy the first of these requirements, especially where the topic is fairly narrowly defined and is discussed by recognized experts. "Infrared Astronomy" (by Robert Gehrz, Gary Grasdalén and John Hackwell), "Neutrino Astronomy" (Raymond Davis Jr), "Stellar Structure and Evolution" (Peter Bodenheimer) and "Supernovae" (David Branch), to pick just a few, are both interesting and authoritative.

The same cannot be said of the many entries supplied by one of the editors and those dealing with topics that are just too broad (astrophysics and cosmology, for example). These just read like fillers and do not provide enough information in the form that an encyclopaedia-reader seeks. Perhaps this compilation reveals its genesis a bit too readily. About half of the

entries (generally the better half) were part of an earlier *Encyclopedia of Physical Science and Engineering*. They can be identified because they were written around 1985 and contain no reference to anything later than that. The rest of the entries have been, well, cobbled together to try to give some kind of broader coverage of astronomy. So instead of getting an up-to-date survey of the whole of the subject by specialists, we have reprints of good but not very recent articles on some topics, together with more recent, but not very inspiring, articles on others (I except the entries dealing with Supernova 1987A here).

I am no expert on many of the subjects covered in the book, and (like most encyclopaedia users) have dipped into it here and there rather than attempting to read it from cover to cover. So I cannot vouch for its overall accuracy or otherwise. Still, Arno Penzias and Robert Wilson will be amused to find themselves described on p.174 as "engineers" who "detected a microwave background coming from all directions in space into a communications antenna they had designed and built for Bell Laboratories". American chauvinism enters into the section on space infrared facilities (p.9), where the US–Dutch–British IRAS satellite is described as the "highly successful NASA IR Astronomical Satellite"; likewise, in a list of future missions, the US SIRTf mission (which is yet to be approved and funded) is described but the European Space Agency's Infrared Space Observatory (ISO, which is now being built and is due for launch in 1993) is not mentioned.

I expect I will find myself using this encyclopaedia; there is a lot of good material here. But it is a pity that the publisher and editors didn't approach their task with a bit more integrity and give us a compilation of more uniform quality. □

Michael Rowan-Robinson is in the School of Mathematical Sciences, Queen Mary College, Mile End Road, London E1 4NS, UK.

Comprehensive sonar imaging of the Easter microplate

R. C. Searle^{**}, R. I. Rusby^{*†}, J. Engeln[‡], R. N. Hey[§], J. Zukin^{||}, P. M. Hunter^{*}, T. P. LeBas^{*}, H.-J. Hoffman[¶] & R. Livermore[#]

* Institute of Oceanographic Sciences, Deacon Laboratory, Wormley, Godalming GU8 5UB, UK

† Department of Geological Sciences, University of Birmingham, B15 2TT, UK

‡ Geology Department, University of Missouri, Columbia, Missouri 65211, USA

§ Hawaii Institute of Geophysics, 2525 Correa Road, Honolulu, Hawaii 96822, USA

|| Institute de Physique du Globe, 4 place Jussieu, 75252 Paris Cedex 05, France

¶ Schloss-Strasse 12, D-8581 Glashuetten, FRG

British Antarctic Survey, High Cross, Madingley Road, Cambridge CB3 0ET, UK

Recent GLORIA and SeaMARC II sidescan sonar surveys have imaged virtually the whole of the Easter microplate, and revealed unprecedented detail and complexity in its tectonic fabric and history. There is clear evidence of rapid microplate rotation, of rapidly evolving plate boundaries and of complex deformation near the northern and southern microplate boundaries.

THE Easter Microplate is about 400 km across, and lies between two roughly parallel arms of the East Pacific Rise (EPR); these are conventionally called the East and West Rifts, although morphologically they include both ridges and rifts. It is bounded to the west and east by the Pacific and Nazca plates (Fig. 1).

The microplate was first recognized from the patterns of seismicity, topography and magnetic anomalies¹⁻³. These suggested the presence of a doubled spreading axis, whereas the northern and southern boundaries were thought to be transform faults⁴. Magnetic lineations near the East Rift fan southwards, implying that its spreading rate increases from north to south¹; the spreading rate on the West Rift then should decrease from north to south. The varying rates on the two rifts should be associated with clockwise rotation of the microplate, making the southern transform boundary transpressive (or 'leaky') and the northern one transpressive⁵. Earthquake focal mechanisms confirm that the East and West Rifts are spreading centres, but show that the northern and southern microplate boundaries are characterized by a mixture of extension, strike-slip and compression^{2,3,5}.

The microplate may have formed when the East Rift developed by northward extension of the southern EPR across a pre-existing, left-stepping transform fault at about chron 2A (3 Myr)⁴⁻⁶. Since then the East Rift has continued to propagate northwards and should have caused the northern boundary of the microplate to migrate with it⁵. The East Rift may eventually supplant the West Rift altogether⁴.

Hey *et al.*⁷, using reconnaissance SeaBeam data, disputed the fanning interpretation because fanning is not observed to the east of the East Rift, and they suggested that the obliquity of magnetic anomalies was mainly the result of tectonic rotation⁸ by several rifts propagating⁹ along the eastern spreading centre¹⁰.

Further SeaBeam^{11,12} and SeaMARC II¹³ work has revealed more detailed structure, mainly of the northern and southern boundaries and their triple junctions with the East Pacific Rise. In particular there is a strong E-W feature at 23° S which was taken^{7,11,12} to be the northern microplate boundary, in spite of a cluster of earthquakes⁵ about one degree farther north.

These studies have contributed to a reasonable general understanding of the microplate, but the controversy over the fanning and propagating rift models of the East Rift remained unresolved. Also, there was still uncertainty about details of the structures of the triple junctions and much of the West Rift, about a possible extension of the microplate north of 23° S, and about the structure and history of the microplate interior. In October–November 1986 we conducted a GLORIA survey of the microplate to address these problems. We have combined the data with the sidescan images from the 1987 SeaMARC II survey¹³ to produce a sidescan mosaic that covers almost all of the microplate (Fig. 2).

Microplate boundaries

We identified the microplate boundaries (Fig. 1, 2) using the sidescan data, together with magnetic and bathymetric data collected concurrently with GLORIA or available from the World Data Center, Boulder, Colorado. Young sea floor was inferred from high acoustic backscatter which indicates a high proportion of unsedimented rock; in addition, spreading axes were inferred from their characteristic magnetic and bathymetric signatures and fault patterns. We recognized propagating rifts by their V-shaped pseudofault patterns and by the presence of rotated spreading fabric⁸. We identified transform and thrust faults by their fault patterns and topographic signatures.

One of our most important findings is that all of the microplate boundaries are evolving rapidly. We have confirmed the presence of a known propagating rift¹⁰ on the East Rift and identified two more along each of the East and West Rifts (Fig. 1). Each of these has propagated to the inside (that is, the microplate side) of its respective 'doomed' rift. Because the absolute direction of ridge 'jumping' is opposite on the two sides of the microplate, the rifts cannot be jumping towards a hotspot unless it is located within the microplate. However, the propagation is tending to minimize the size of the microplate (which may help it to rotate more easily against the compressive constraints from the major plates at its northern and southern borders), and also tends to minimize the total length of the microplate boundary and therefore probably also the total energy dissipation. An important corollary is that the time-averaged accretion of the West and East Rifts is asymmetric (slower on the microplate than on the major plates), even though instantaneous spreading may be fairly symmetric.

Further evidence for rapid boundary evolution is seen in the three transforms of the West Rift. At none of these is a strong fracture zone preserved far beyond the actively slipping section, implying that the transforms formed relatively recently. Where short fossil traces are seen (most notably at the western end of the 23° S transform) they strike obliquely to the current slip direction, implying rapidly changing relative motions.

At the northern end of the East Rift (23° S) the axis is located in Pito Deep^{11,12}. Between there and the propagating rift tip near 23.5° S we cannot identify the axis unambiguously. At

* Present address: Department of Geological Sciences, University of Durham, South Road, Durham DH1 3LE, UK.

23.3° S, acoustic backscatter, bathymetry and magnetics are all consistent with its occupying a highly asymmetric position in a graben at 111.87° W, although it could be up to 40 km farther east.

On the West Rift the axis south of the propagating rift at 25.3° S seems to be a simple ridge as far as 26.4° S¹¹. From there Francheteau *et al.*¹¹ suggested that it is offset eastwards through a series of grabens into the southern margin of the microplate (Orongo fracture zone) at 26.8° S. The GLORIA and SeaMARC data, however, suggest a spreading axis (high backscatter accompanying fine, closely spaced lineaments approximately normal to the spreading direction) extending south-eastwards to 26.8° S, 114.9° W, through a gap in the 2,000 m ridge that bounds the south side of the south-west rift. We think this is either the active tip of the south-eastward propagating West Rift, or one that was recently abandoned as the extension jumped eastward to the location favoured by Francheteau *et al.*

The northern and southern microplate boundaries (Pito and Orongo fracture zones¹¹) are complex structures subject to both shear and compression⁵. Short segments adjacent to the divergent eastern and western boundaries may be pure strike-slip structures¹¹, but over most of their lengths they seem to be complex transpressive zones, often with substantial relief^{7,11}, and possibly comprising en echelon thrust blocks, particularly in the north¹². Our data confirm this, and in addition show Orongo fracture zone to be associated with NW-SE striking faults that may be oblique normal faults similar to those seen in some Atlantic fracture zones and indicative of broadly distributed shear^{14,15}.

The sidescan data confirm the existence of a large overlapping spreading centre¹² at the southern end of the East Rift, but reveal a confused zone of poorly defined tectonic fabric between it and Orongo fracture zone to the west. The precise position of the southern triple junction is still not clear, though it is probably near or south of a line joining Orongo fracture zone and the overlapping spreading centre¹².

At the northern triple junction, our data confirm a transform trending 088° to the west of the EPR, and a lineament of similar trend to the east. This cannot be a pure ridge-fault-fault junction^{7,11} however, because two colinear transforms are inconsistent with known¹⁶ or plausible Pacific-Nazca-Easter motions: there must either be another plate or deformation of at least one of the known plates (Fig. 3).

We see such deformation in the Nazca plate adjacent to the northern edge of the microplate. The EPR-parallel spreading

fabric here trends almost due north, 10° anticlockwise from the normal to the predicted^{16,17} Pacific-Nazca spreading direction. Moreover, the spreading rate on the EPR between 21° S and 23° S is less than predicted¹⁶. The difference implies generally northward compression in the corner of the Nazca plate between the northern EPR and the microplate, which is also the sense of predicted^{5,16} Easter-Nazca motion there. This part of the Nazca plate may thus be in the process of being incorporated into the Easter microplate (Fig. 3).

A cluster of compressive earthquakes between 22° S, 114° W and 23° S, 112° W was thought to mark the site of this deformation⁵. The GLORIA and SeaMARC data show a line of en echelon E-W faults associated with large ridges, which we interpret as thrust blocks. Several of these are also sites of dextrally curving spreading fabric of the type normally found at dextral-offset transform faults¹⁴, suggesting that a component of shear is also present.

Microplate interior

In the microplate interior both the overall level of acoustic backscattering and the general trends of tectonic lineaments are highly variable. It is possible, however, to define domains tens of kilometres across within which these features are much more uniform. Generally such domains have sharp edges, across which both the backscatter level and the strike of lineaments change abruptly. Within each domain the tectonic lineaments are straight, parallel and closely spaced, and we interpret most of them as relict spreading fabric. This pattern might indicate that the microplate was built up from many independently moving smaller blocks. A simpler explanation is that most of the domain boundaries are pseudofaults, and that the domains are small blocks of differently aged lithosphere that have been transposed and rotated^{8,9} by propagating rifting. Part of our current work is directed towards reconstructing this complex evolution.

The most obvious domain boundaries are the two inner pseudofaults from the propagating northern tip of the East Rift and southern tip of the West Rift. The former curves south-westwards across the microplate from near 23.5° S, 112° W to 25° S, 115° W. At its northern end there is a sharp contrast in the acoustic backscatter across it, reflecting a discontinuity in the age of the sea floor. This contrast diminishes south-westwards as the sea floor on both sides ages and collects more sediment. By comparison with nearby areas traversed by GLORIA outside the microplate, we infer that the age contrast

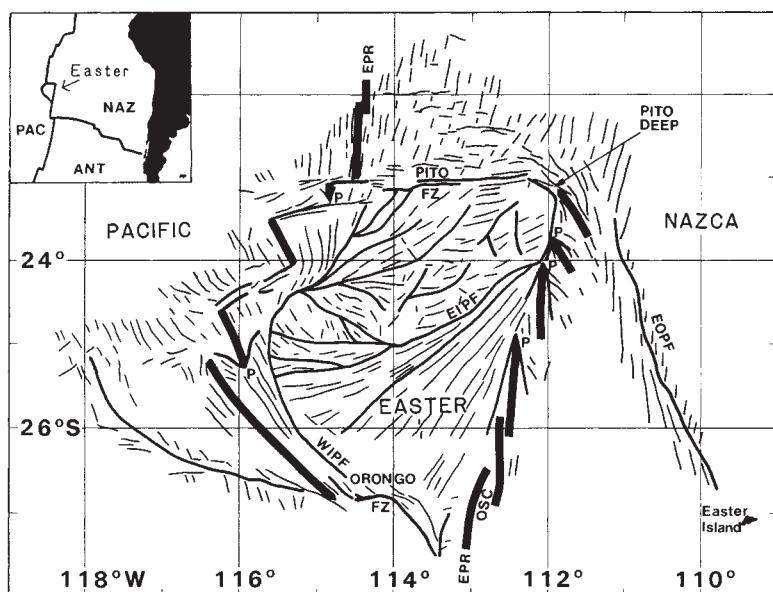


FIG. 1 Simplified tectonic interpretation of Easter microplate based on GLORIA and SeaMARC II sidescan sonar data (see Fig. 2). Heavy lines show plate boundaries. Medium lines mark pseudofaults and other discontinuities in crustal accretion. Light lines are representative fault scarps of the tectonic spreading fabric. P indicates a propagating rift tip; WIPF, EIPF and EOPF are the West Rift inner and East Rift inner and outer pseudofaults, respectively; FZ indicates a fracture zone; OSC indicates an overlapping spreading centre.



FIG. 2 Sidescan sonar mosaic of Easter microplate. Most of the area is covered by GLORIA data (40-km-wide swaths). SeaMARC II sidescan data¹³ (10-km-wide swaths) are used to provide complete coverage of the area around Pito Deep¹¹ (north-east corner of microplate boundary) and the southern end of the south-west rift, and to partially fill other areas of

incomplete GLORIA coverage. Darker tones show stronger acoustic backscattering, which usually indicates less sediment cover and therefore younger sea floor. Data have been digitally processed to correct for geometric distortions, to equalize the effective gain at different ranges, and to remove line dropouts.

at the northern end of the eastern inner pseudofault is several million years. There is a marked change in strike of the tectonic lineaments across the pseudofault, approaching 90° in places.

The most dramatic feature of the sonar mosaic is the very sharp, curved boundary between high and low backscattering at what we interpret as the inner pseudofault of the West Rift, just to the east of its axis. The evidence of an age discontinuity across this feature, combined with the continuously varying angle between it and the West Rift spreading fabric, and its southwards approach to the West Rift axis, indicate that this is a pseudofault. Magnetic anomalies and the backscattering contrast suggest that it is younger than its eastern counterpart. This evidence of relatively recent propagation of its tip, and the existence of two other propagators on the West Rift, suggest that it is less moribund than has sometimes⁴ been thought.

Between the East Rift's inner pseudofault and its current spreading axis, the spreading fabric displays a fanned pattern, at least to first order. A few pseudofaults produced by the other propagating rifts along the East Rift can be inferred, but their expression is quite subtle. The inner pseudofault of the 25° S propagator¹⁰ is revealed between 25.0° S and 25.5° S by a backscatter contrast and a 10° change in strike of the spreading fabric across it, but generally the others can be detected only by subtle ($<10^\circ$) changes of strike. Thus, although the passage of propagating rifts other than the East Rift tip might account for some of the tectonic rotation of the East Rift spreading fabric and magnetic anomalies, the majority seem to have been produced as simple fanning caused by the continuously changing spreading rate along the rift.

North and west of the East Rift inner pseudofault, the microplate interior is much more complex, and although individual domains can still be recognized, the changes between them are less consistent. The oldest part of the microplate appears to be near 24.5° S, 115.5° W. It has the lowest acoustic backscattering,

and its tectonic fabric is the least clearly preserved; both of these features indicate thick sediment cover. The 3.5-kHz records over the area indicate sediment thicknesses of ~ 10 m. Immediately to the north-east of this domain is a fan of lineaments pointing west. This may be a relict propagating rift.

The West Rift inner pseudofault becomes less clear north of this domain. Possibly the relatively recent southward propagation of the West Rift tip began about here, perhaps at the 24.5° S transform. In the north-west corner of the microplate the currently active 23.5° S transform seems to give way eastwards to a succession of NE-SW-trending fabric, the history of which we have not yet deciphered.

The tectonic fabric in the north-east corner of the microplate runs almost E-W. It is interrupted in places by N-S trending 'discordant' zones, which are generally characterized by topographic ridges and curving offsets of the tectonic fabric (such as that near 23.5° S, 112.5° W). In some ways this area resembles the deforming corner of the Nazca plate due north of the microplate, but rotated through 90° , the N-S discordant zones being analogous with the E-W thrust features farther north.

Microplate history

A record of the microplate's history is preserved in the tectonic fabric of its interior and in the adjacent parts of the Pacific and Nazca plates.

East of the microplate, we followed the initial outer pseudofault towards Easter Island. GLORIA, SeaBeam¹² and SeaMARC II show that its northern end is near 22.9° S, 111.3° W, ~ 50 km east of the north end of the East Rift. Northward propagation seems to have stalled near that position, with the rift tip recently jumping westwards¹². We were able to trace the pseudofault south to 26.3° S, 110.0° W, where it clearly marks a 15° change in trend and distinct apparent age contrast in the spreading fabric. It probably continues closer to Easter Island, and may even cross it to reappear just to the south-east of that feature, although our evidence there is less clear. Nevertheless, it seems that the suggestion^{4,18} that the formation of Easter Island is closely related in time and space to the initiation of the East Rift may be essentially correct.

As expected, the inner and outer initial pseudofaults of the East Rift are mirror images, and details of their shape, such as the more northerly strike near 24.3° S (marking a brief acceleration in propagating or deceleration in spreading rate), are matched from one to the other. Our preliminary reconstruction suggests that the East Rift began to open ~ 5 Myr ago, some 2 Myr earlier than previously^{4,18} thought.

We have limited new data between the present East Rift axis and the outer pseudofault, but this and archived magnetics suggest a history of fairly uniform spreading, with no major discontinuities of spreading-axis direction and little or no fanning. The Pacific plate, however, shows rather confused fabric. There are several sets of curving trends indicating minor spreading-axis offsets and, near 25° S, 117 – 118° W, some apparent age discontinuities that suggest a continued history of rapid evolution of the West Rift for the past several million years.

We searched for a spreading fabric discontinuity west of the microplate that would mark its initiation, but found no clear-cut evidence of one. We therefore think it likely that the West Rift evolved continuously as the microplate formed.

We also investigated the major topographic ridge^{7,11} that runs from 26.2° S, 117° W to 27.0° S, 114° W. Between 116° W and 118° W, E-W lineaments strike west away from this ridge, and we interpret these as the fossil traces of a multi-transform¹⁹ fracture zone that was probably the original southern transform boundary of the microplate⁴⁻⁶.

We suggest the microplate may have been initiated as follows. There seems to be a critical spreading rate on the EPR near the Easter microplate, above which transforms do not occur²⁰. This may be because they are unstable at speeds for which the lithospheric strength falls below the transform's shear resist-

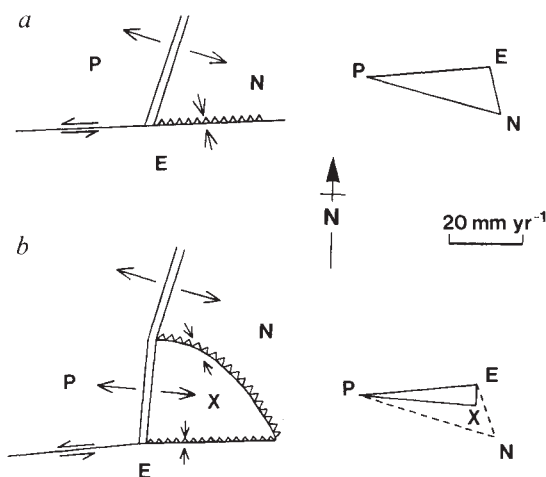


FIG. 3 *a*, Predicted¹⁰ RFT (ridge-fault-trench) plate boundaries (left) and velocity triangle (right) for the northern microplate-EPR (Easter-Pacific-Nazca, E-P-N) triple junction. P-E and P-N diverge by 22° . *b*, Left: observed configuration at the northern triple junction. Right: velocity triangle assuming there is another plate X or deformation of the Nazca plate north of the microplate; we assumed P-X motion to be the observed¹⁰ EPR spreading rate of 132 mm yr^{-1} at 22.6° S in a direction of 094° , normal to its observed trend and that of the structures produced by it over the past 0.75 Myr. This predicts almost north-south convergence across E-X at the triple junction. P-E and P-X cannot be colinear transforms unless the EPR is spreading 10° obliquely, and we suggest that the east-west structure observed east of the triple junction is the fossil trace of the 23° S transform. X lies close to E-N: if the corner of the Nazca plate were gradually being incorporated into the microplate by deformation, points on X should track along line N-E in velocity space; the present position of X indicates that this process is over halfway complete near the triple junction.

ance²¹. We speculate that ~5 Myr ago the sinistral-offset transform near the present southern boundary of the microplate was near its stability limit. Some event then pushed it over the limit: a small increase in spreading rate (perhaps accompanying increased discharge at the Easter mantle plume and incidentally producing Easter Island) would have critically weakened the lithosphere; alternatively, a small clockwise change of Pacific-Nazca motion, such as occurred at 3.4–3.9 Myr (ref. 22), would have made the transform transpressional and increased its resistance to slip. The resulting stresses would have made the East Rift begin to propagate and form a rotating microplate²³, causing the southern transform to open up and the West Rift to propagate along it⁵.

Schouten and others²⁴ have presented a description of microplate kinematics in which they liken the motion of a microplate to that of a ball bearing turning between major plates. Their analysis, which predicts that both east and west initial outer pseudofaults should strike E–W, refers only to the instantaneous case; in reality, the propagating rifts leave oblique pseudofaults,

and the microplate grows by accretion at the East and West Rifts. As it turns, therefore, the microplate acts more like a cam than a ball bearing, applying N–S pressure to the major plates. The Easter microplate is thus deforming the Nazca plate to its north.

The rotation emphasized in Schouten's model must be accompanied by strong vorticity in the asthenosphere. Indeed, in this region where the hot, fast-spreading lithosphere is thin and weak^{4,7}, the fluid motion of the asthenosphere may be dominant, and the observed brittle-plate kinematics of the sea floor may be largely a passive response to it. The strong clockwise vorticity associated with the initial transform would have continued after that transform became unstable, and been directly converted into rotation of the microplate.

Viewed at a regional scale, the microplate has produced a pattern of lineaments remarkably similar to a whorl in a fluid vortex (Fig. 2). We may here be coming close to a limit where rigid-plate tectonics and continuum-fluid mechanics are equally valid and useful descriptions of lithospheric behaviour. □

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- Herron, E. M. *Nature* **240**, 35–37 (1972).
- Forsyth, D. W. *Earth planet. Sci. Lett.* **17**, 189–193 (1972).
- Anderson, R. N., Forsyth, D. W., Molnar, P. & Mammertick, J. *Earth planet. Sci. Lett.* **24**, 188–202 (1974).
- Handschemacher, D. W., Pilger, R. H., Foreman, J. A. & Campbell, J. F. *Mem. geol. Soc. Am.* **154**, 63–76 (1981).
- Engeln, J. F. & Stein, S. *Earth planet. Sci. Lett.* **68**, 259–270 (1984).
- Okal, E. A. & Cazenave, A. *Earth planet. Sci. Lett.* **72**, 99–116 (1985).
- Hey, R. N. *et al. Nature* **317**, 320–325 (1985).
- Searle, R. C. & Hey, R. N. *J. geophys. Res.* **88**, 6433–6447 (1983).
- Hey, R. N., Duennel, F. K. & Morgan, W. J. *J. geophys. Res.* **85**, 3647–3658 (1980).
- Naar, D. F. & Hey, R. N. *J. geophys. Res.* **91**, 3425–3438 (1986).
- Francheteau, J. *et al. Earth planet. Sci. Lett.* **89**, 363–374 (1988).
- Zukin, J., Francheteau, J. & Armijo, R. *Eos* **69**, 1423 (1988).
- Hey, R. N. & Naar, D. F. *Cruise Report, RV Moana Wave, 8711* (Hawaii Institute of Geophysics, University of Hawaii, Honolulu, 1987).

- Searle, R. C. *J. geol. Soc. Lond.* **143**, 743–756 (1979).
- Searle, R. C. & Laughton, A. S. *J. geophys. Res.* **82**, 5313–5328 (1977).
- Naar, D. F. & Hey, R. N. *Am. Geophys. Un., Geophys. Monogr.* (in the press).
- Minster, J. B. & Jordan, T. H. *J. geophys. Res.* **83**, 5331–5354 (1978).
- Hagen, R., Baker, N. A., Naar, D. F. & Hey, R. N. *Mar. geophys. Res.* (submitted).
- Searle, R. C. *Geology* **11**, 607–610 (1983).
- Naar, D. F. & Hey, R. N. *Geology* (in the press).
- Oldenburg, D. W. & Brune, J. N. *J. geophys. Res.* **80**, 2575–2585 (1975).
- Harbert, W. & Cox, A. J. *geophys. Res.* **94**, 3052–3064 (1989).
- Gallo, D. G. *Eos* **63**, 446 (1982).
- Schouten, H., Klitgord, K. D. & Gallo, D. G. *Eos* **69**, 488 (1988).

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Three-dimensional crystal structures of *Escherichia coli met* repressor with and without corepressor

John B. Rafferty, William S. Somers, Isabelle Saint-Girons* & Simon E. V. Phillips

Astbury Department of Biophysics, University of Leeds, Leeds, LS2 9JT, UK

*Unité de Biochimie Cellulaire, Institut Pasteur, 28 Rue du Docteur Roux, 75724 Paris, Cedex 15, France

The three-dimensional crystal structure of *met* repressor, in the presence or absence of bound corepressor (*S*-adenosylmethionine), shows a dimer of intertwined monomers, which do not have the helix-turn-helix motif characteristic of other bacterial repressor and activator structures. We propose that the interaction of *met* repressor with DNA occurs through either a pair of symmetry-related α -helices or a pair of β -strands, and suggest a model for binding of several dimers to *met* operator regions.

THE gene regulatory control system for the biosynthesis of methionine in *E. coli* was one of the first to be discovered¹. Methionine, a key amino acid in protein synthesis, is the precursor

to *S*-adenosylmethionine (SAM), which functions as the main intracellular methyl donor. SAM is the corepressor bound by the methionine repressor protein, one component of the regulatory system. The *Met* repressor is the product of the *metI* gene, and represses its own gene and those coding for enzymes involved in methionine and SAM synthesis².

The determinations of the three-dimensional structures of proteins involved in transcriptional regulation in *E. coli* and its phages show that they have a common structural motif, formed by a pair of α -helices linked by a tight turn, in otherwise distinctly different structures. This has been called the 'helix-turn-helix' motif, and has been implicated in the sequence-specific DNA-protein binding of the phage λ cI repressor protein and the cro protein^{3,4}, the related phage 434 repressor and Cro proteins^{5,6}, the *E. coli* cyclic AMP-binding protein (CAP)⁷ and the *E. coli trp* repressor⁸. In these dimeric proteins the second helices, or 'recognition helices', of symmetrically related motifs have been postulated to insert into adjacent turns of the major groove of approximately B-form DNA. This has been confirmed directly

by the crystal structures of the DNA complexes of the *trp* repressor⁹, the phage 434 repressor and the Cro protein^{10,11} and λ cI repressor¹².

We report here the crystallographic structures of the *met* repressor in the presence and absence of bound SAM, determined at 1.9- and 1.7-Å resolution, respectively. The *met* repressor is a dimer comprised of two highly intertwined monomers, which do not contain helix-turn-helix motifs. On the basis of model-building experiments we suggest ways in which the protein might recognize its DNA target through two symmetry-related α -helices or a pair of β -strands.

The *metJ* gene has been cloned, its nucleotide sequence determined¹³, and the repressor protein overexpressed and purified¹⁴. It is a polypeptide of 104 amino acids of relative molecular mass (M_r) 11,996 and exists as a stable dimer in solution. Two molecules of SAM bind non-cooperatively to each *metJ* dimer, thereby increasing its affinity for DNA markedly¹⁵.

The *met* biosynthetic genes under the control of the *met* repressor are scattered throughout the chromosome over at least six loci². An examination of the operator sequences reveals an underlying 8-base pair (bp) repeating unit, referred to as a 'met box', in two to five tandem copies.

In an attempt to explain the variation of operator length and the results of biochemical and genetic experiments¹⁵, we propose a model that involves the binding of several repressor dimers to tandem sites in the operator regions.

Structure determination

Crystals of the aporepressor were grown from PEG600 (polyethylene glycol of average M_r 600) by the hanging-drop vapour-diffusion method¹⁶. Native X-ray diffraction data were collected

to a resolution of 1.7 Å on an area detector system (Xenotronics) and phased using isomorphous and anomalous 3 Å derivative data collected on a diffractometer (Enraf-Nonius CAD4) (Table 1). Initial electron-density map fitting was carried out at 3 Å resolution by using the program FRODO on a computer graphics system (Evans and Sutherland PS300). Density corresponding to the polypeptide backbone was strong and clear, but was improved by averaging about the dimer 2-fold axis, and solvent flattening^{17,18}. After the initial model-building stage the 2-fold averaging was discontinued and the structure subjected to restrained least-squares refinement¹⁹, resulting in the current *R*-factor of 0.21 for all data in the range 10–1.7-Å resolution.

Crystals of SAM-bound repressor were grown using microdialysis, by dialysing out Mg^{2+} ions. Data were collected to 1.9 Å resolution on an area detector system (Xenotronics) and the structure was determined by molecular replacement. Aporepressor coordinates were used as a starting point for rotation- and translation-function searches, with unambiguous solutions found for both functions. Electron-density maps were generated based on calculated phases, and model building and refinement carried out as for the aporepressor. Two SAM molecules were located as positive difference density peaks in the maps. To avoid bias towards the starting model, segments of the structure in regions of poorly defined density were removed and the maps were recalculated. The *R*-factor now stands at 0.22 for data in the range 10–1.9 Å resolution.

Repressor structure

For both forms of the repressor, the electron density map is clear from the N-terminus to the C-terminus. The map reveals two highly intertwined monomers creating the dimer. Each

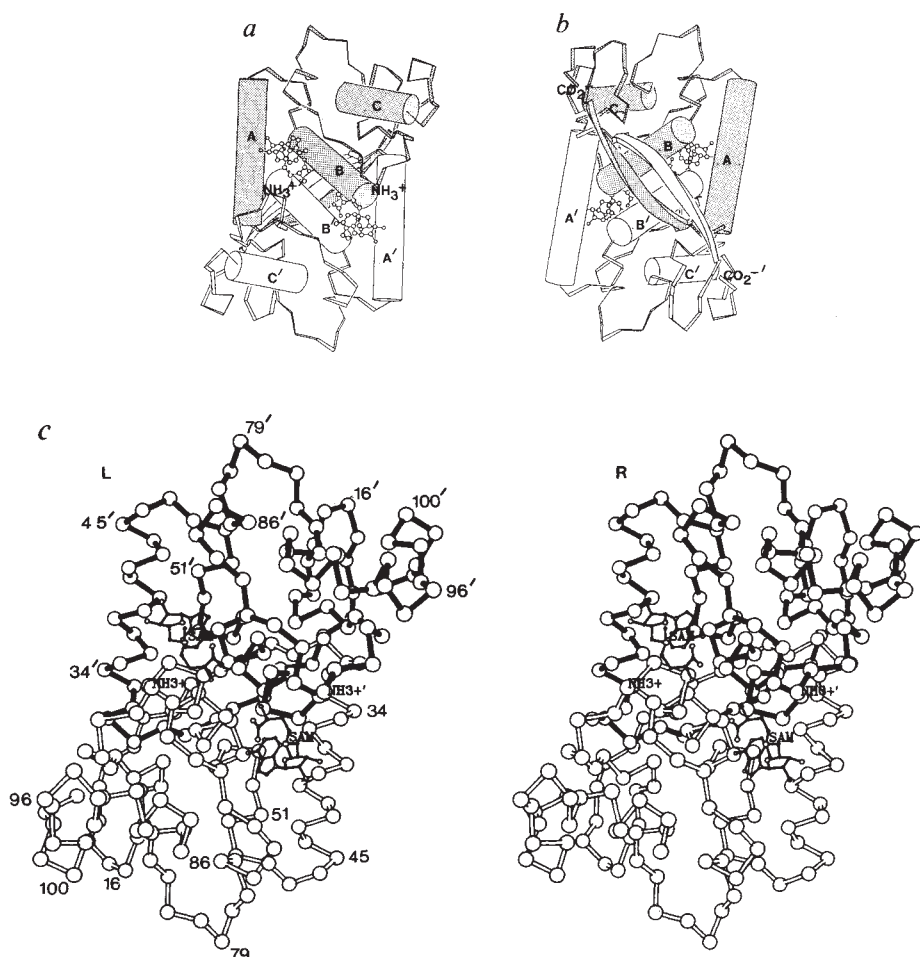


FIG. 1 SAM-bound *met* repressor. *a, b*, Dimer with helices represented as cylinders, β -strands as thick arrows and rest of chain as ribbon. One monomer is displayed shaded. *a*, View along dimer dyad axis into face containing C-helices. *b*, View along dyad axis in opposite direction to *a*, showing intertwined β -strands. Helices are labelled A, B, and C (A', B' and C') and the termini NH_3^+ and CO_2^- (NH_3^+ and CO_2^-). *c*, View is a stereo α -carbon backbone trace of the dimer as 'ball and spoke' in the same direction as *a*; one monomer has open bonds and the other shaded bonds. Selected residues are numbered in one view, the presence or absence of a prime symbol differentiating the monomers. SAM is shown in all-atom form. Aporepressor structure is not shown, but apart from the SAM and the flexible loops, which are remote from the proposed DNA-binding region, it would be indistinguishable. The first flexible loop (His 14-Glu 19) is immediately before the β -strand and the second (Lys 78-Ser 81) between helices B and C close to the first loop in space.

TABLE 1 Crystal data

	Crystallography conditions	Space group	Unit cell	N_{ref} *	$R(I)$ †	R ‡	Resolution range	r.m.s. dev§	Solvent molecules
Aporepressor	PEG600 100 mM PO_4^- 100 mM Na acetate pH 7.0	$P2_1$, $z=2$	$a=35.6$ Å $b=62.6$ Å $c=44.5$ Å $\beta=102.4^\circ$	19,850	0.045	0.21	10→1.7 Å	0.03, 0.07	85
Repressor-SAM	Mg^{2+} 15 mg ml ⁻¹ 2.5 mM SAM 10 mM Na cacodylate buffer, pH 6.2	$P3_221$, $z=6$	$a=75.0$ Å $b=75.0$ Å $c=82.0$ Å	18,378	0.067	0.22	10→1.9 Å	0.03, 0.08	107
Heavy-atom derivative data									
	Reagent	N_{ref}	resolution range	$R(I)$ ¶	$R(F)$ *	$R(Fhle)$ **	$\langle m \rangle$ ††		
	0.5 mM PCMBs (24 h soak)	3,561	25→3.0 Å	0.023	0.26	0.39	0.63		

Met repressor protein concentration ~10 mg ml⁻¹; PO_4^- , mixed sodium-potassium phosphate buffer; PCMBs, sodium *p*-chloromercuribenzenesulphonic acid; and z , number of dimers in the unit cell (non-crystallographic dimers in both crystal forms).

* N_{ref} , total number of independent reflections. It represents 80% and 95% of available data for the SAM complex and apo-forms, respectively, in the resolution ranges quoted.

† $R(I)$ is $\sum_{hkl} |I_i - I_m| / \sum_{hkl} I_m$, where I_i and I_m are the observed intensity and mean intensity of related reflections, respectively.

‡ R is $\sum_{hkl} ||F_o| - |F_c|| / \sum_{hkl} |F_o|$, where $|F_o|$ and $|F_c|$ are the observed and calculated structure factor amplitudes, respectively.

§ Deviation of the bond lengths and the 1–3 bond angle distances respectively from accepted standard values.

|| Solvent molecules, number of water molecules added to the later stages of refinement. They were selected by the presence of small, approximately spherical regions of positive electron density as seen in difference density maps at possible hydrogen-bonding distances from donor or acceptor groups on the protein.

¶ $R(I)$ is $R(I)$ for centric data only.

* $R(F)$ is $\sum_{hkl} |F_d - F_n| / \sum_{hkl} |F_n|$, where F_d and F_n are the derivative and native structure factor amplitudes, respectively.

** $R(Fhle)$ is $\sum_{hkl} |F_{hle} - F_c| / \sum_{hkl} |F_{hle}|$, where F_{hle} is the lower estimate derived from the observed data, and F_c the calculated value, for the structure factor due to the heavy atom.

†† Mean figure of merit for relative phase accuracy over all reflections.

monomer is composed of three α -helices (A-helix, residues 30–45; B-helix, residues 52–66; C-helix, residues 86–94) and a β -strand (residues 20–29), which account for 50% of the polypeptide; the remainder consists of turns or extended chain (Fig. 1). The *met* repressor does not possess a helix-turn-helix motif.

The dimer contacts are made along adjacent faces of the 2-fold related B-helices and by the β -strands. The antiparallel β -strands loop over one another and firmly establish the intertwined dimer. Substantial inter-monomer contacts have also been observed for *trp* repressor⁸, where the predominantly α -helical monomers intertwine. Symmetry-related interactions occur between the N-terminal end of the B-helix and the β -strand of the other monomer, and between the C-terminal ends of the B-helices.

Examination of the dimer interface explains a spontaneous point mutation²⁰ that leads to non-functional repressor—a change of the Ala 60 codon to a Thr codon. This change results in a reduction of the levels of the dimer obtained² and suggests an increased accessibility to proteolytic cleavage. We propose that the mutation hinders dimerization, because residue 60 lies in the interface between the B-helices.

The inner B-helix is flanked by the A-helix, which links it to the β -strand. Side-chain interactions between A-helices and B helices fix their relative orientations. A stretch of open chain,

including an eight-residue loop, joins the B-helix to the C-helix. A network of hydrogen-bonded side chains links this loop and the side chains and backbone of the C-helix, thereby determining its position.

Throughout both structures 2-fold symmetry is well maintained. The only main departures arise at the turns (His 14–Glu 19) leading to the β -strands, and can be accounted for by differences in the crystal lattice environment. An examination of the two structures shows that all four equivalent regions are the most variable parts of the protein and, by implication, very flexible. The other notable departure from 2-fold symmetry occurs in the aporepressor structure at a loop (Lys 78–Ser 81); here the side chain of Lys 78 bridges the loop in one monomer, but the side chain of Arg 80 bridges it in the other monomer. The latter arrangement is also seen in the loops in the SAM-bound structure.

Corepressor binding

S-adenosylmethionine binds to the *met* repressor dimer at two independent, symmetrically related sites, as seen in the crystal structure. This confirms the results of equilibrium dialysis experiments², which indicated that SAM binds to the dimer with a stoichiometry of 2:1. The binding sites are located close to the 2-fold dimer axis and on the same face of the protein as the

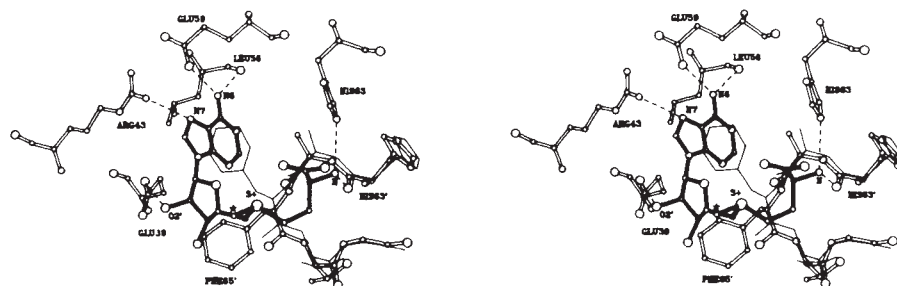


FIG. 2 Stereo view of a SAM molecule-binding site. Protein is in open ball-and-spoke representation for SAM-bound form (=) and as simple shaded stick representation for apo-form (—), showing movement of the side chain of Phe 65'. A SAM molecule is shown in shaded ball-and-spoke representation and hydrogen bonds denoted by dashed lines. The two monomers are differentiated by presence or absence of a prime symbol after residue numbers and the activated methyl group of SAM marked with an asterisk.

C-helices. The binding of SAM involves the insertion of its purine ring into a hydrophobic pocket on the protein surface. Comparison with the aporepressor structure shows that this pocket is otherwise occupied by the phenyl ring of Phe 65', which is swung out of the way when SAM is bound (Fig. 2). The activated methyl group of SAM results in a trivalent sulphur atom which carries a positive charge. Closer examination of the location of the binding site shows that it is at the C-terminal end of the B-helix, with the sulphur <3.4 Å from the carbonyl oxygen of Ala 64. The helices are dipolar and the positive sulphur is thus positioned near the net negative C-terminal end.

The hydrophobic pocket, into which the purine ring is inserted, is formed by the side chains of Leu 70, Ala 64', Phe 61', Leu 56, the peptide plane that joins Ala 64' and Phe 65', and interactions from the side chains of Glu 59 and Arg 43. There is no 'adenine recognition loop' as proposed by Remington *et al.*²¹ for citrate synthase. Each SAM molecule makes symmetric hydrogen-bonded contacts with both monomers (Fig. 2). The contacts are formed between the adenosyl and methionine moieties of SAM, and the backbone and side chains of the A- and B-helices. Hydrogen bonds from the carbonyl oxygen of Leu 56 and side-chain carboxyl oxygen of Glu 59 to N6, in addition to one from the guanidino group of Arg 43 to N7, help to position the purine ring. A further contact is made between O2 of the C3'-endo-ribose ring and the carboxyl group of the side chain of Glu 39. Finally, the amino nitrogen, N, of the methionyl moiety contacts the ring N₂ of His 63 and the carbonyl oxygen of the related residue His 63' in the other monomer.

There does not seem to be any major change in the dimer conformation on the binding of SAM. A comparison of the structures for both forms of the repressor, by least-squares fitting of their equivalent α -carbons using the program PUBFIT, shows an excellent superimposition, apart from the flexible loops mentioned above. The r.m.s. deviation for the fitting of the α -carbons of aporepressor to SAM-bound repressor, excluding the flexible loops, was 0.43 Å, compared with 0.34 Å and 0.34 Å for the fitting of the non-crystallographically related monomers to each other in the aporepressor and SAM-bound repressor dimers, respectively.

DNA binding model

The binding of the *met* repressor occurs at several operator regions of different lengths on the *E. coli* chromosome². But underlying this variation is the repeating 8-bp met box unit. Its

consensus sequence, dAGACGTCT, has been determined by sequence analysis of the operator regions and is an inverted repeat. There are integral numbers of met boxes (between two and five) upstream of the genes that are controlled by *met* repressor. A comparison of the natural operators to the consensus reveals a trend of a decrease in homology as the number of met boxes increases¹⁵. In operators that contain more than two met boxes the homology to the consensus is usually higher in the central boxes. Genetic studies, such as operator constitutive mutations in the operators of *E. coli metF*, *metC* (ref. 15) and the *Salmonella typhimurium metB*²², as well as filter-binding experiments in which synthetic sequences are used, have confirmed that repeating met box sequences constitute specific targets for the binding of repressor.

Structural studies of other repressor systems have indicated that dimers of similar size to *met* repressor contact between 14 and 18 bp of essentially B-form DNA. Recent structures of protein-DNA complexes^{9,10,12,23,24} show that DNA distorts on the binding of protein, but it is not possible to predict what distortions, if any, might occur in the met boxes. NMR data from a 16-bp segment that is closely homologous to two met boxes is consistent with that of B-form DNA in solution (J. Parkinson, personal communication). We therefore modelled a 32-bp segment of ideal B-form DNA, composed of four met boxes (for example, the length of the *metA* operator) with a 10.5 bp helical repeat, on a computer graphics system. An initial assumption was made that the 2-fold axis of the dimer would coincide with that between two met boxes, such that each half of the dimer experienced an identical environment when bound to the operator. Note that symmetry can be maintained if the dimer axis is considered coincident with the central 2-fold axis of a met box, and the binding interaction is thus offset by 4 bp relative to the first alignment. In either alignment there remain only two degrees of freedom in modelling the overall geometry of the repressor-operator interaction, excluding the possibility of changes in the structure of the DNA and/or the protein on binding. These degrees of freedom are the relative separation of the protein and the DNA, and their relative rotation about their common 2-fold axis. The orientation of protein and DNA were initially chosen so that the face of the protein that contains the C-helices approached two adjacent turns of the major groove and one central turn of the minor groove. Because of the prominent nature and relative separation (35 Å) of the C-helices, we propose that they form part of the binding site for DNA in an analogous way to that observed for the helix-turn-helix motif.

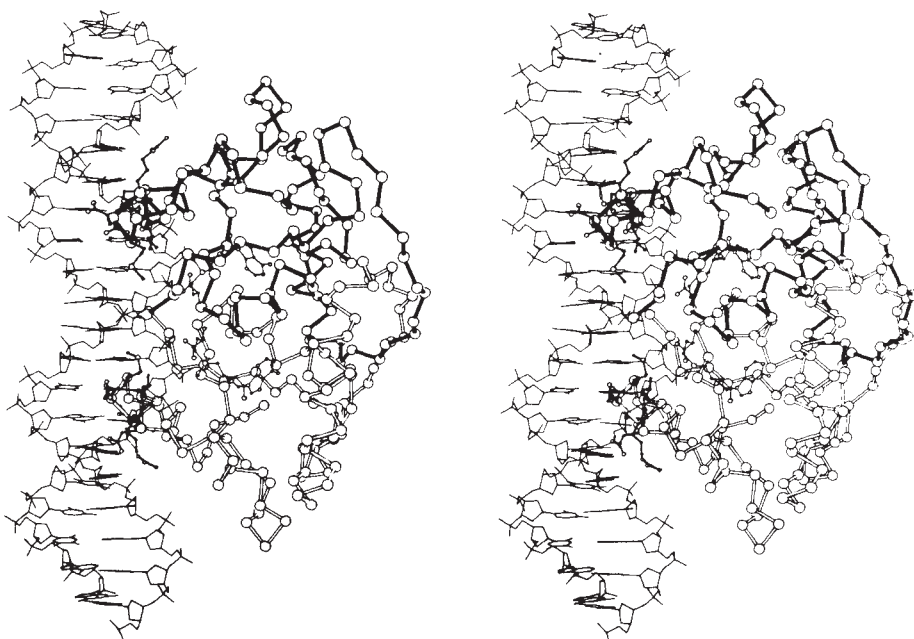


FIG. 3 A stereo pair showing a single dimer binding with inserted C-helices to a piece of B-form DNA comprised of repeating met boxes as generated on a computer graphics system. The dyad axis of the dimer is coincident with the 2-fold axis between met boxes. DNA is shown in stick form, the protein monomers as an α -carbon trace in open and shaded ball-and-spoke form and the SAM and residues Glu 90, Ile 91, Arg 93, Glu 94 and Met 95 as all atom. In the model shown, the fit of the C-helices in the major groove has been maximized; this has, however, resulted in the first three N-terminal residues approaching the DNA phosphate backbone too closely. The alternative model with the β -strands inserted into the major groove can be imagined by translation of the repressor dimer 'through' the DNA (that is, to the left) along the mutual dyad axis, followed by a small clockwise rotation about this axis.

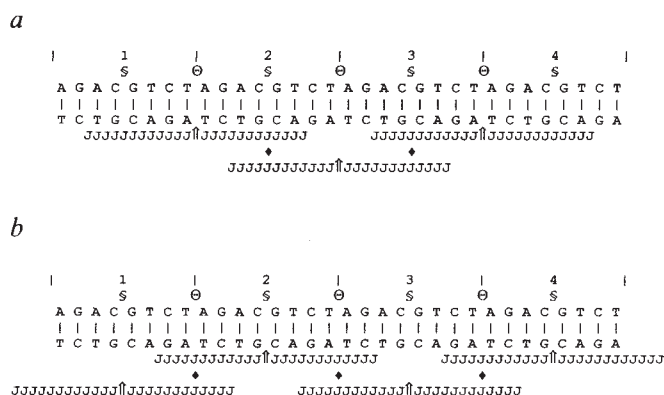


FIG. 4. Schematic representations of the model for multiple repressor binding to met boxes. Shown are four perfect consensus met boxes (1–4), to which are bound MetJ dimers (JJJJ) at sites centred 8-bp apart. *a*, Dimer dyad axes coincident with axes between met boxes. *b*, Dimer dyad axes coincident with 2-fold axes within met boxes. Axes between the met boxes are shown by Θ and within the met boxes by $\S$. Dimer dyad axes are shown by arrows and the 2-fold axes between dimers by solid diamonds.

Docking of the protein and DNA was done manually using interactive computer graphics; the C-helices were readily inserted into the major groove to form a very good fit (Fig. 3).

This simply fitted model showed a good overall surface complementarity, and the possibility of specific contacts between the base-pair edges and protein side chains in the manner proposed by Seeman *et al.*²⁵. The C-helices lie approximately parallel to the major groove but with their carboxy termini penetrating more deeply into it. Several hydrogen bonds could be formed between the side chains of residues Glu 86, Lys 89, Glu 90, Arg 93 and Glu 94 of the C-helix, and the bases and phosphate backbone of the DNA. There could also be a favourable van der Waals' contact formed between the side chain of

Ile 91 and the base-pair edges. Further similar hydrogen bonding and van der Waals' contacts could also be made by the first two residues at the N-terminus, Ala 1 and Glu 2, and by the side chains of residues Asp 72, Arg 80, Met 95 and Asn 98 to form a substantial interface.

An alternative arrangement can also be modelled in which the antiparallel β -strands on the opposite face of the dimer are inserted into the major groove. This model does not show as good a complementary fit to the DNA; the buried surface area of protein and DNA in the complex is $208 \text{ \AA}^2$ compared with $585 \text{ \AA}^2$ for the model with inserted C-helices, expressed as solvent accessibility²⁶ using a probe of radius $1.4 \text{ \AA}$. But this alternative model cannot be discounted as it has identical symmetry to the previous model and is sterically feasible. Experiments using site-directed mutagenesis are at the moment being performed to determine which of these binding models represents the DNA-protein interaction in solution.

The binding of corepressor causes little conformational change in the repressor structure as mentioned above. This is in contrast to the situation with the tryptophan repressor, where binding of the co-repressor causes the helix-turn-helix motifs to move markedly further apart²⁷ and increases affinity of the repressor for DNA. A similar allosteric mechanism has been proposed for the activation of CAP by the binding of cAMP (ref. 7), but it would not seem to be the case for the methionine system. The possibility exists, however, that the act of crystallization of the protein could have locked the aporepressor and repressor-SAM complex into the different lattices with fortuitously similar conformations, although there is no evidence to substantiate this hypothesis.

Our model with inserted C-helices places SAM in the protein-DNA interface. SAM fills a gap between the surfaces of the protein and the DNA, and a possible contact could be made between the methionyl moiety of SAM and the phosphate backbone. The corepressor is positioned between the net negative C-terminus of the B-helix and the negatively charged phosphate backbone of the DNA, and its positively charged sulphur

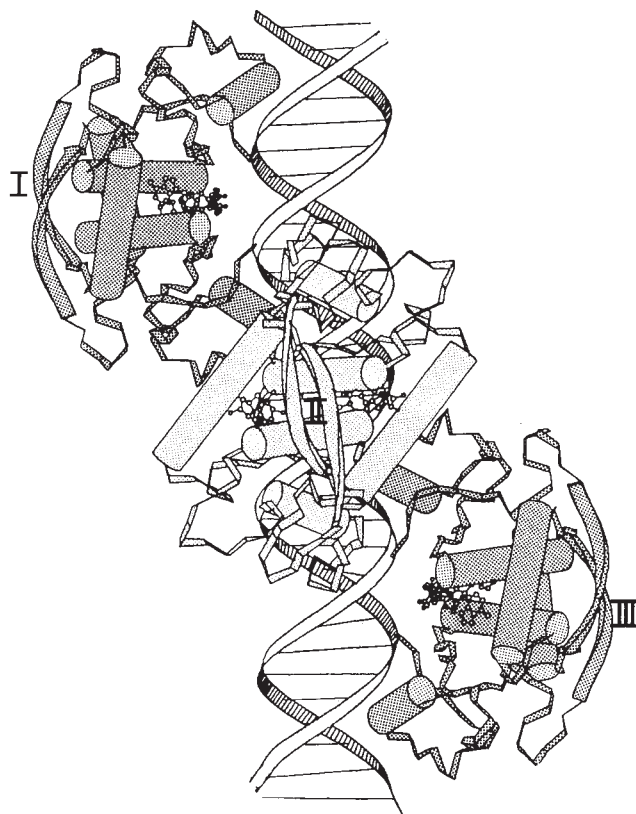


FIG. 5. Diagram of the multiple repressor binding model for three MetJ dimers bound with inserted C-helices to a 32-bp segment of B-form DNA represented as a helical ladder. The sides are formed by the deoxyribose phosphate backbone and the rungs by the base pairs. Dimers are labelled I, II and III forming a left-handed helix, with an 8-bp rise per dimer and a relative rotation about the DNA of $\sim 85^\circ$.

functions perhaps as a counter-ion. This feature could offer an explanation of how SAM functions to increase DNA affinity and why the close structural homologue *S*-adenosylhomocysteine, which lacks the activated methyl and hence the positive charge on the sulphur, is inactive as a co-repressor (I. Parsons *et al.* manuscript in preparation). How SAM could function in the model with inserted β -strands is not immediately obvious.

In the light of recent structural determinations of repressor-operator complexes, we hesitate to propose a more detailed interpretation of the interactions. We have obtained a crystal form of a *met* repressor-operator complex suitable for X-ray-structure determination, which in conjunction with site-directed mutagenesis experiments, should provide the details of the protein-DNA interaction.

Model of binding involving several dimers

An intriguing feature of the *met* regulatory system is the action of the repressor on operators with various numbers of tandemly repeated met boxes. DNase protection experiments involving the *metF* operator¹⁵ show coordinate protection of all five met boxes that is a total of 40 bp, by the *met* repressor. We believe that several repressors must bind to such operators¹⁵. It could be that a repressor dimer merely contacts one 8-bp met box, as observed for our model with inserted β -strands, but our favoured model with inserted C-helices indicates a binding site of nearer 16 bp. This poses the problem of why a dimer seems to protect odd numbers of 8-bp met boxes. Detailed biochemical and genetic experiments with both natural and synthetic operator sites indicate that repressor binds cooperatively to sites of tandem repeats of 8 bp units¹⁵.

We propose here a structural model that involves the binding of repressor dimers to overlapping sites in the *met* operators. A similar proposal has been made for the *trp* repressor system²⁸, although the idea has been opposed on the basis of results from stoichiometry measurements²⁹. Our postulated arrangement can accommodate binding to various numbers of met boxes, including the number present in the *metF* operator. Because of the 2-fold symmetry at the centre of met boxes as well as between them, however, exact positioning of the *metJ*-encoded dimer can be achieved in either of two alignments outlined above,

while maximizing the use of available symmetry (Fig. 4). In alignment one (Fig. 4a) the dimer axis coincides with the 2-fold axis between met boxes, and in alignment two (Fig. 4b), it coincides with the central axis of a box, resulting in n met boxes binding $n-1$ or n dimers, respectively.

In more detail, a repressor is proposed to bind and enhance subsequent repressor binding at sites centred 8 bp apart along the DNA. Increased affinity for additional repressor binding may result from protein-protein contacts between the turns (Glu 94-Gly 96) following the C-helices of adjacent dimers in the major groove of the DNA (Fig. 5). Possible non-covalent contacts could occur between the side chain of Met 95, the main-chain atoms of the turn and the N-terminal residues, and those from the related residues in an adjacent dimer. These contacts may account for the cooperativity of operator binding¹⁵.

This model can also explain the additional 2-fold symmetry in a met box which would coincide with the 2-fold axis between adjacent protein dimers in alignment 1 (Fig. 4a) or the dimer 2-fold axis in alignment 2 (Fig. 4b).

When viewed in terms of a 3-D model (Fig. 5), the dimers form a left-handed superhelix around the DNA with an 8 bp rise per dimer and a rotation of $\sim 85^\circ$. The model permits this overlapping of sites and juxtaposition in the major groove of the C-helices from adjacent dimers without steric clashes, while a reasonable fit of these helices in the major groove is maintained. A similar model could be made for binding through the β -strands, which would allow possible cooperative interactions without steric clashes between dimers centred 8 bp apart.

Although this is only a model, it provides explanations for various observations in binding experiments and uses all the available symmetry in the system. Experiments are underway to test the validity of the idea. $\square$

Note added in proof: We have recently solved the crystal structure of a repressor-operator complex, which shows two Met-repressor dimers bound to a synthetic 19-mer oligonucleotide containing two met boxes. The dimers bind in alignment 2, with their β -strands inserted into the major groove. They form a left-handed superhelix around the DNA, with protein-protein interactions between them, as predicted in this paper.

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- Cohen, G. N. & Jacob, F. *C. R. Acad. Sci., Paris* **248**, 3490-3492 (1959).
- Saint-Girons, I., Parsot, C., Zakin, M. M., Bärzu, O. & Cohen, G. N. *CRC Critical Reviews in Biochemistry* **23**, (CRC, Cleveland, Ohio, 1988).
- Pabo, C. O. & Lewis, M. *Nature* **298**, 443-447 (1982).
- Anderson, W. F., Ohlendorf, D. H., Takeda, Y. & Matthews, B. W. *Nature* **290**, 754-758 (1981).
- Mondragon, A., Subbiah, S., Almo, S. C., Drott, M. & Harrison, S. C. *J. molec. Biol.* **205**, 189-200 (1989).
- Mondragon, A., Wolberger, C. & Harrison, S. C. *J. molec. Biol.* **205**, 179-188 (1989).
- McKay, D. B., Weber, I. T. & Steitz, T. A. *J. biol. Chem.* **257**, 9518-9524 (1982).
- Schevitz, R. W., Otwinowski, Z., Joachimski, A., Lawson, C. L. & Sigler, P. B. *Nature* **317**, 782-786 (1985).
- Otwinowski, Z. *et al. Nature* **335**, 321-329 (1988).
- Aggarwal, A. K., Rodgers, D. W., Drott, M., Ptashne, M. & Harrison, S. C. *Science* **242**, 899-907 (1988).
- Wolberger, C., Dong, Y., Ptashne, M. & Harrison, S. C. *Nature* **335**, 789-795 (1988).
- Jordan, S. R. & Pabo, C. O. *Science* **242**, 893-899 (1988).
- Saint-Girons, I., Duchange, N., Cohen, G. N. & Zakin, M. M. *J. biol. Chem.* **259**, 14,282-14,285 (1984).
- Saint-Girons, I. *et al. J. biol. Chem.* **261**, 10,936-10,940 (1986).
- Phillips, S. E. V. *et al. Nature* **341**, 711-715 (1989).

- Rafferty, J. B. *et al. J. molec. Biol.* **200**, 217-219 (1988).
- Bricogne, G. *Acta Crystallogr.* **A32**, 832-847 (1976).
- Wang, B. C. *Meth. Enzym.* **115**, 90-112 (1985).
- Hendrickson, W. A. & Konert, J. H. *Biomolecular Structure, Function, Conformation and Evolution* Vol. 1 (ed. Srinivasan, R.) 43-57 (Pergamon, Oxford, 1981).
- Liljestrand-Golden, C. A. & Johnson, J. R. *J. Bact.* **157**, 413-419 (1984).
- Remington, S., Wiegand, G. & Huber, R. *J. molec. Biol.* **158**, 111-152 (1982).
- Urbanowski, M. L., Plamann, L. S. & Stauffer, G. V. *J. Bact.* **169**, 126-130 (1987).
- McClarin, J. A. *et al. Science* **234**, 1526-1541 (1986).
- Suck, D., Lahm, A. & Oefner, C. *Nature* **332**, 464-468 (1988).
- Seeman, N. C., Rosenberg, J. M. & Rich, A. *Proc. natn. Acad. Sci. U.S.A.* **73**, 804-808 (1976).
- Lee, B. & Richards, F. M. *J. molec. Biol.* **55**, 379-400 (1971).
- Zhang, R.-g. *et al. Nature* **327**, 591-597 (1987).
- Kumamoto, A. A., Miller, W. G. & Gunsalus, R. P. *Genes Dev.* **1**, 556-564 (1987).
- Klig, L. S., Carey, J. & Yanofsky, C. *J. J. molec. Biol.* **202**, 769-777 (1988).

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Cooperative tandem binding of *met* repressor of *Escherichia coli*

Simon E. V. Phillips*, Iain Manfield†, Isobel Parsons†‡, Barrie E. Davidson§‡, John B. Rafferty*, William S. Somers*, Danielle Margarita§, Georges N. Cohen§, Isabelle Saint-Girons§ & Peter G. Stockley†

* Astbury Department of Biophysics, and † Department of Genetics, University of Leeds, Leeds LS2 9JT, UK

§ Unité de Biochimie Cellulaire, Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France

We present biochemical and genetic data to support the hypothesis that the *Escherichia coli met* repressor, MetJ, binds to synthetic and natural operator sequences in tandem arrays such that repression depends not only on the affinity of the DNA-protein interaction, but also on protein-protein contacts along the tandem array. This represents a novel form of regulatory switch. Furthermore, there seems to be homology between the organization of the *met* and *trp* operators.

MANY gene regulatory events are mediated by the interaction of several proteins, which simultaneously make contacts with each other and specific DNA sequences¹. Bacteriophage λ CI repressor, for example, binds cooperatively to operator sites O_R1 and O_R2, each dimer of repressor contacting adjacent DNA sequences and a neighbouring repressor. Activation of eukaryotic transcription seems to require the interaction of a series of DNA-binding proteins, many of which are in contact with each other as well as with DNA, and act together to facilitate the binding of RNA polymerase². In this issue Rafferty *et al.*³ report the determination, by X-ray crystallography to high resolution, of the crystal structure of the *E. coli* methionine aporepressor, MetJ, and its complex with the corepressor, S-adenosyl-methionine (SAM). A model for the binding of repressor to DNA is also proposed, and it is suggested that tandem binding accounts for features of the natural *met* operators. Here we present genetic and biochemical evidence that supports this hypothesis and implies that transcription of genes in the methionine biosynthesis pathway is controlled not only by protein-DNA interactions, but also by protein-protein contacts formed between adjacent repressor dimers along the DNA duplex. This model for the interaction of MetJ and DNA explains the biochemical data and is consistent with the results of the structural studies.

Methionine biosynthetic pathway

The biosynthesis of methionine is accomplished by a series of transformations from other key metabolites⁴. Several stages of the pathway are common to the biosynthesis of other major metabolites, such as diaminopimelate, lysine, threonine and isoleucine, and the pathway culminates in the activated methyl cycle, a key metabolic control point. Flux through this pathway is mainly controlled by the product of the *metJ* gene, the MetJ repressor, which acts at at least six operators dispersed throughout the genome. The activity of MetJ is regulated not by methionine, but by another product of the pathway, SAM^{5,6}. The cell must therefore control the levels of transcription of

selected genes in a pathway used not solely for methionine biosynthesis, by sensing the level of a central metabolite, SAM. A clue to the possibility that the repressor does not act by a simple series of on/off switches is provided by an analysis of the 5' flanking regions of the regulated genes. This reveals an eight-base pair (bp) tandem repeat or 'met box', with a consensus repeat sequence of dAGACGTCT (ref. 7; Table 1). The number of met boxes varies considerably from two in *metC*, to five in *metF* and *metB*. The shortest naturally occurring operator (*metC*) has two met boxes and could represent the minimum operator length. There are no perfect met-box consensus sequences in the natural *E. coli* operators, although a single perfect met box has been reported in the *metR* operator of *Salmonella typhimurium*⁸.

Probing operator-repressor interactions

To test whether repressor can bind to a perfect consensus operator *in vitro*, we cloned a synthetic 16-mer oligonucleotide corresponding to two tandem met boxes into the *Sma*I site of the plasmid pUC18, and used the operator insert flanked by the polylinker sequence in filter-binding and gel-retardation studies of DNA-protein interaction. Figure 1a shows the result of a filter-binding assay in which this consensus operator was used in the presence or absence of SAM. The protein showed a very pronounced enhancement of binding to the consensus in the presence of SAM (half-maximal binding $\approx 4.5 \times 10^{-8}$ M) and binding did not reach saturation in the absence of SAM. The binding curve in the presence of SAM is distinctly sigmoidal, indicating cooperative binding. Scatchard plots of the binding data (data not shown) also indicate positive cooperativity. Similar behaviour has been observed for other repressor proteins that bind DNA as dimers but which can dissociate into monomers at low concentration¹. Monomers of *met* repressor,

TABLE 1 Operator consensus sequences

Consensus	AGACGTCT	AGACGTCT	AGACGTCT	AGACGTCT	AGACGTCT
<i>metC</i>	AGACaTcC 75%	AGACGTaT 87.5%			
<i>metB</i>	AtACGcaa 50%	AGAaGTtT 75%	AGAtGTcC 75%	AGAtGTaT 75%	tGACGTcC 75%
<i>metF</i>	cttCaTCT 50%	ttACaTCT 62.5%	gGACGTCT 87.5%	GaACGgaT 62.5%	AGAtGTgc 62.5%
<i>metA</i>	AGctaTCT 62.5%	gGAtGTCT 75%	AaACGTaT 75%	AagCGTaT 62.5%	
<i>metE</i>	gGAtGaaT 50%	AaACTtGc 50%	cGcCtTcC 50%		
<i>metR</i>	AGgatTtT 50%	AGcCGTcC 75%	AGAtGTtT 75%	AcACaTcC 62.5%	

The met box sequences from six *E. coli met* operators²⁴ have been aligned and a consensus sequence deduced. As explained in the text, the minimum size of the operator is expected to be that of two such met boxes, that is 16 bp. The sequences of the operators are shown together with their percentage homology to the consensus (indicated under each met box). Upper-case letters denote residues homologous to those of the consensus. Note that in general the homology to the consensus is best in the shorter operators, and towards the centre of the longer ones.

‡ Present addresses: Biochemistry Department, University of Melbourne, Parkville, Victoria 3052, Australia (B.E.D.); Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06520, USA (I.P.).

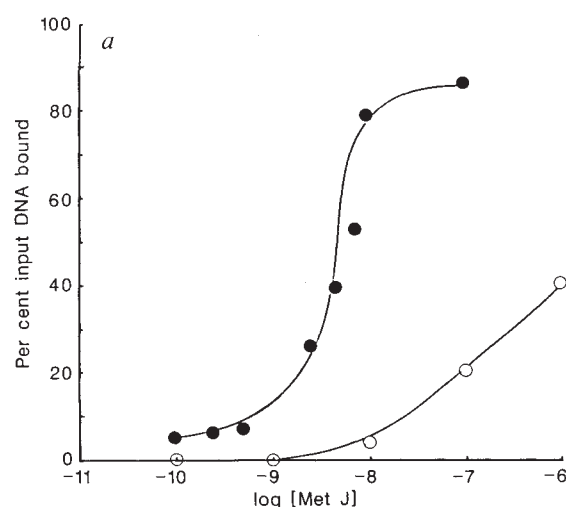
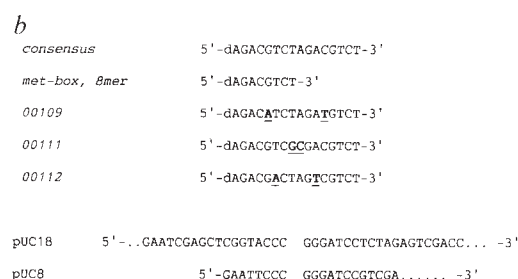


FIG. 1 Filter-binding of operator constructs. Synthetic oligonucleotides comprising the perfect 16-mer consensus sequence, the 8-bp half-site and sequence variants, were ligated into *Sma*I-cleaved pUC18, which was then used to transform *E. coli* TG1 to the ampicillin-resistant phenotype on 5-bromo-4-chloro-3-indolyl- β -galactopyranoside/isopropyl- β -D-thiogalactopyranoside plates. Plasmid DNA from white colonies was sequenced to confirm the presence of the desired insert. The polylinker containing the operator site was then excised by digestion with *Eco*RI or *Hind*III (or both) and radiolabelled with Klenow polymerase and [α - 32 P]dNTPs, followed by the second digest and purification over polyacrylamide gels. The 78-bp



radiolabelled fragment was then used in a nitrocellulose filter-binding assay²⁵ (I.P., P.G.S. and J. Parkinson, manuscript in preparation). Essentially, varying concentrations of MetJ were complexed with the target DNA fragment in 0.1 M KCl, 10 mM Tris-HCl, 0.5 mM EDTA, pH 8.0, at 37 °C for 60 min before filtering. The control pUC18 polylinker fragment lacking any insert produced no detectable binding even at the highest protein concentrations ($\sim 10^{-6}$ M). **a**, Filter-binding of the perfect consensus 16-mer sequence in the presence (●) or absence (○) of 0.1 mM SAM. **b**, Sequences of the synthetic operator sites, together with the polylinker sequences into which they were cloned in either pUC8 or pUC18. Variations from consensus are underlined.

however, are extensively intertwined³, a feature seen in the *trp* repressor⁹ and which presumably accounts for the large amount of free energy required to dissociate *trp* repressor dimers¹⁰.

As well as a perfect consensus of two met boxes, we tested a construct containing a single met box. This construct had a markedly lowered affinity for repressor; it failed to saturate at protein concentrations at which binding is sequence-specific, as did a series of sequence variants (Fig. 1b) in which residues throughout the central region of the 16-bp consensus were changed, including those at relatively conserved positions. All the sequence changes result in operators with dramatically reduced affinities for repressor (data not shown); in the presence of corepressor they bind less strongly to repressor than does the perfect consensus even in the absence of corepressor, although all the operators show SAM dependency. These data indicate that the two met-box 16-bp site can act as an efficient operator,

that two base-pair changes in the 16-mer oligonucleotide can have a profound effect on the affinity of the protein for DNA and also that bases at the centre of the 16-mer are important for repressor binding.

We carried out a similar series of experiments in which site-directed mutants of the natural *metC* operator were used. The *in vivo* phenotypes of the mutated *metC* operators were analysed by using a *lacZ* reporter gene. The *metC* operator contains two met boxes with three sequence differences from a perfect consensus. With a single exception, stepwise modification of the sequence resulted in a small increase in repression activity with every alteration towards the consensus sequence, and a distinct drop in activity with mutations away from the consensus (Fig. 2a).

To determine precisely which regions of the perfect consensus-pUC18 polylinker are bound by the repressor, we carried

<i>a</i>	Allele	Sequence	Operator function(%)
	Con.	AGACGTCTAGACGTCT	
	<i>metC</i>	AGACATCCAGACGTAT	100
	1004	AGACATCCAGACGTAT	10
	1015	AGACATCCAGACGTCT	121
	1005	AGACGTCCAGACGTAT	87
	1008	AGACATCTAGACGTAT	143
	1058	AGACGTCTAGACGTAT	181
	1558	AGACGTCTAGACGTCT	219

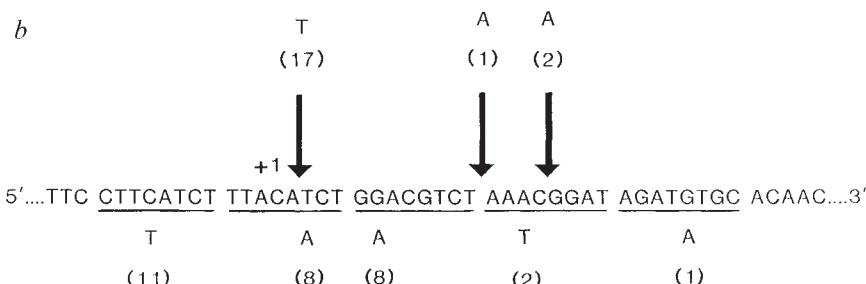


FIG. 2 Mutagenesis and *in vivo* repression of the *metC* and *metF* operators. **a**, Analysis of *metC* mutants *in vivo*. Natural *metC* operator region was ligated into a *lacZ* reporter construction (pDIA16) and then used to transform *E. coli* *metJ*⁺ and *metJ* strains (TP2100 and GT1008, respectively²⁶) to the ampicillin-resistant phenotype. *In vivo* repression ratios were measured as the ratio of the β -galactosidase activity in the two isogenic strains. The β -galactosidase activity for the repressor mutant strain is 32 nmol per min per mg protein at 27 °C, whereas that for the wild-type strain is 2.1. They represent the average values of three independent β -galactosidase assays which are quite reproducible. Operator function has been defined as a

percentage by equating the repression ratio observed for the wild-type fusion to 100% and a repression ratio of one (that is, no repression) to 0% operator function. Underlined bases indicate differences from the consensus. **b**, Nucleotide sequence of the *metF* regulatory region showing the position of point mutations and their effect on operator function. Replacement and insertion mutations are indicated below and above the sequence respectively. The percentage operator functions are shown in parentheses. The met boxes 1–5 are indicated by solid bars and are separated by spaces. Transcription of *metF* commences at the C residue marked +1.

out both DNase I protection¹¹ and methylation protection-interference¹² experiments (results summarized in Fig. 3a). The nuclease footprint is roughly centred on the consensus 16-mer oligonucleotide, but extends to either side of it by about 8 bp. The methylation experiments also revealed protein contacts outside the consensus region. Similar results for nuclease protection were obtained with the natural *metC* operator.

The dimensions of the repressor protein indicate that these protection patterns could not be generated by a single MetJ dimer unless the DNA is wrapped around the protein, which seems unlikely in view of the small radius of such a complex. Such a model seems even less likely when the results of nuclease protection assays on larger natural operators are considered (see below); the simplest interpretation is that several protein molecules are bound.

We tested for this binding of several protein molecules by performing gel retardation assays^{13,14} (Fig. 3b). Test fragments were retarded in the presence of repressor protein and SAM. Even at the lowest protein concentrations at which binding occurs, up to three retarded species (c1-c3) were observed, which were converted to the most retarded band, c3, with increasing protein concentration. This is consistent with the formation of a series of protein-DNA complexes, each differing by the addition of further repressor dimers. All experiments involving DNase I and methylation protection were performed at concentrations at which the chief species present would be c3, indicating that c3 at least is a complex that contains multiply bound proteins. Similar experiments involving several perfect met-box sequences tandemly arranged and cloned into the plasmid pUC8 showed that there is an increase in the numbers of retarded complexes with increasing numbers of met boxes, such that an increase in the size of the operator site by one extra met box (8 bp) results in one extra retarded complex (data not shown). Preliminary analysis indicates that increasing the number of met boxes decreases the protein concentration at which retardation to larger complexes occurs, consistent with cooperative repressor binding (I.M. and P.G.S., manuscript in preparation).

The binding of several repressor dimers can also be demonstrated for the longer natural operators, such as *metF* which has five met boxes. Analysis of a series of mutant *metF* operators is shown in Fig. 2b. Point mutations and insertions (indicated by arrows) are shown, together with the operator function (in parentheses) as a percentage of wild-type function (100%), again measured from *lacZ* fusions. It can be seen that point-mutational changes throughout the five met boxes of the *metF* operator can lead to dramatic (10- to 100-fold) reductions in the activity of the repressor *in vivo*. Operators with mutations towards the perfect consensus had repression ratios that were higher than those of wild-type operators. But the most striking result from these data comes from an analysis of point insertions in the operator sequence. In particular, point insertions at positions between +13/14 and +17/18 lowered the measured repression rates by 100-fold or 50-fold, respectively. The start of transcription lies 5' to these met boxes, and effective repression might be expected should only met boxes 1 and 2, or 4 and 5 be bound. A simple interpretation of these data is that binding of MetJ repressor dimers to this operator is cooperative, and depends on protein-protein contacts being made which are disrupted by base insertions, whereas DNA sequence changes merely alter the intrinsic affinity of the DNA-protein interaction between the operator and a single repressor dimer. This hypothesis is consistent with DNase I footprinting of the *metF* operator revealing coordinate protection across the entire 40 bp¹⁵.

Cooperative tandem-binding model

To account for these data in a consistent manner we propose a model for the interaction, which predicts the protection of several 8-bp sites by the binding of repressor dimers to tandem

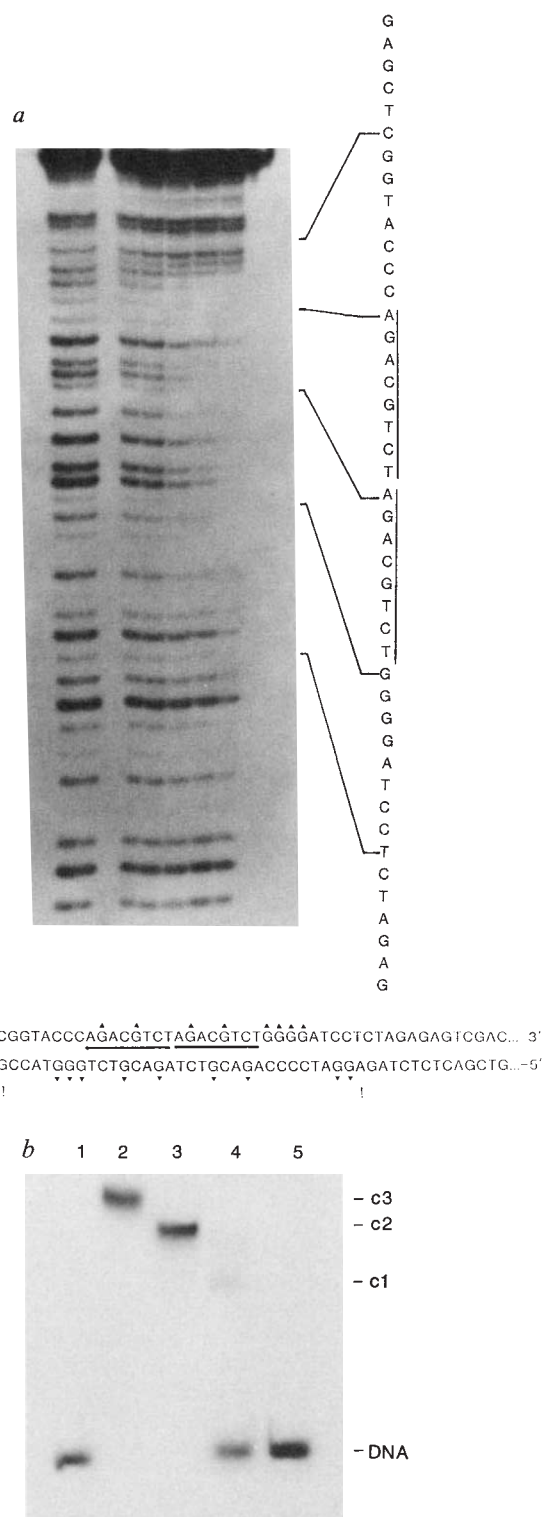
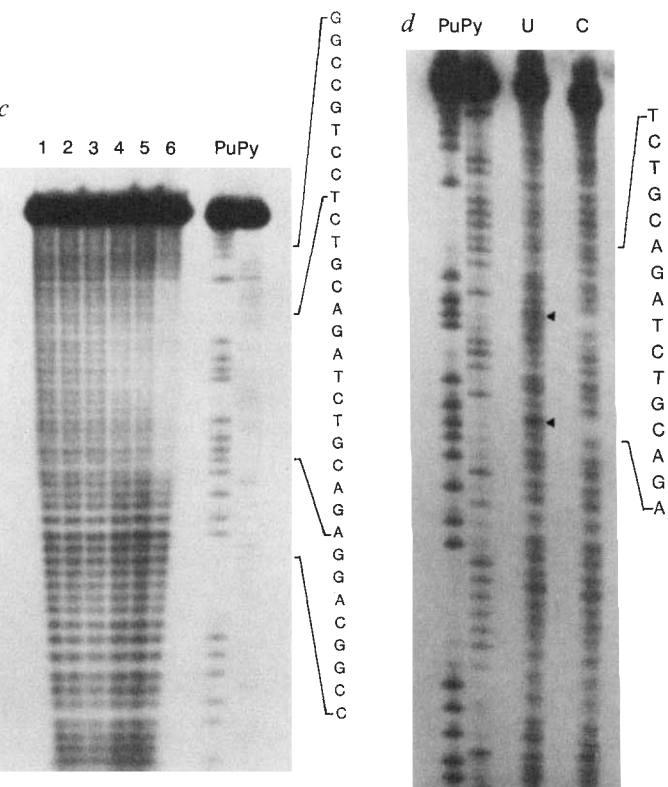
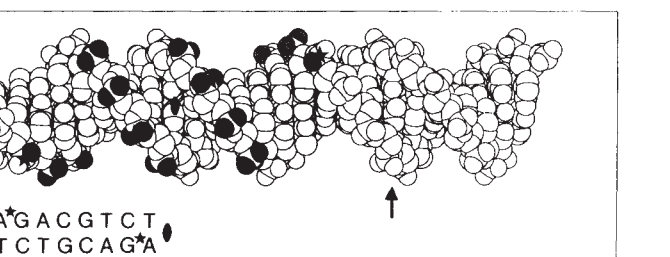


FIG. 3 Tandem binding of repressor proteins. *a*, Autoradiograph (top) of DNase I digestions of the pUC18-16mer construct (bottom strand) in the presence of increasing amounts of repressor. Lane 1, no MetJ; lane 2, 3.0×10^{-9} M MetJ; lane 3, 6.0×10^{-9} M MetJ; lane 4, 12.0×10^{-9} M MetJ; lane 5, 25.0×10^{-9} M MetJ; lane 6, 50.0×10^{-9} M MetJ; lane 7, 100×10^{-9} M MetJ. Below is a summary of DNase I and DMS protection of the complex formed between MetJ and the perfect pUC18-16mer construct (underlined). The limits of nuclease protection on the bottom strand are indicated by an exclamation mark, and bases protected from methylation by $\blacktriangle$. *b*, Gel retardation of pUC18-16mer consensus construct. Gels were run at pH 7.0 in 10 mM phosphate buffer and contained 0.1 mM SAM (I.M. and P.G.S., manuscript in preparation). Lane 1, no MetJ; lane 2, 0.5×10^{-6} M MetJ; lane 3, 0.5×10^{-8} M MetJ; lane 4, 0.5×10^{-10} M MetJ; lane 5, 0.5×10^{-12} M MetJ.

alignments for the placement of repressors along the DNA, which indicate different stoichiometries for repressor-met-box interactions; that is, for alignment 1 we predict a stoichiometry of $n - 1$ repressor dimers bound per n met boxes, whereas for alignment 2 we predict n repressor dimers per n met boxes. The analysis becomes more difficult if overlaps into flanking sequences occur, as they clearly do in the experiments with the synthetic consensus and in the *metC*-operator. We have designed an 'anti-met-box' 8-bp sequence in which we incorporated at each position in the equivalent met box the least frequently occurring



★GACGTCT
CTGCAGA★



MetJ repressor (100 nM) and the resultant mixture analysed by gel retardation. Unshifted (U) and complexed (C) DNA was then eluted from the gel, modified phosphates were cleaved and then analysed on a 20% polyacrylamide gel alongside a Maxam and Gilbert sequencing ladder (top strand is shown). The two phosphates where ethylation leads to complete exclusion from the repressor-operator complex are indicated by arrows. *e*, Summary of footprinting and protection data on the 32-bp anti-met-box construct shown as a space-filling representation of an idealized 10-fold B-DNA duplex. The extent of the region protected from MPE cleavage (~ 18 bp) is indicated by arrows. The non-esterified phosphate oxygens which lose more than 10% of their surface accessibility based on the model of the complex shown in Fig. 3 of ref. 3 (this issue) are indicated by shading. ★, Phosphates identified in Fig. 4*d*: ●, central dyad between the two met boxes.

nucleotide as determined from the conservation table (Table 1). To discriminate between the two alignments we constructed an operator fragment containing the perfect 16-mer consensus oligonucleotide embedded symmetrically between two such anti-met-boxes; this construction might be expected to minimize binding in the flanking sequences. Gel retardation analysis of this construct reveals a single retarded species under conditions that produce the largest retarded complex in the pUC18 construct (Fig. 4b). At very high repressor concentrations, even this construct resulted in three retarded species. A simple interpretation of these data is that the single retarded species is a complex between a single repressor dimer and the DNA fragment. Symmetry arguments would then suggest that the repressor 2-fold axis of symmetry must be aligned with the unique 2-fold axis of symmetry between met boxes. We have determined the region footprinted by the repressor in this complex by using the reagent methidium-propyl-EDTA-Fe (MPE)¹⁶ (Fig. 4c). Densitometry of autoradiographs of these footprints is consistent with a short region of protein-DNA interaction centred, on a helical projection of the operator site, about the sequence 5'-dCTAG-3', that is, about the junction between the met boxes.

Constructs produced with the alternative binding site, that is, a 4-bp shifted sequence and its equivalent anti-met-box site, have lower affinities for MetJ, as judged by both filter-binding and gel retardation assays, than do the sites described here (data not shown).

To confirm the position of the repressor dyad relative to those in the operator, we performed phosphate-ethylation interference experiments¹² on the complex formed with the anti-met-box construct (Fig. 4d, e). The positions of close contact between protein and DNA identified in this way show excellent correlation with other complex structures determined by X-ray crystallography¹⁷. Four such phosphates are identified in the MetJ-SAM-anti-met-box construct. These are shown projected onto a DNA helix in Fig. 4e. As expected from the MPE-footprinting data, the contacts are symmetrically disposed across the dyad between the two met boxes. The size of the ethylation footprint is consistent with the binding of a single dimer at this site. Surface accessibility calculations of the model in which a single MetJ dimer is docked to a B-form DNA duplex via the C-helices³ (in alignment 1), correlate well with the protection of these four phosphates. We note, however, that the alternative β -strand model could account for these results if two repressor dimers were bound according to alignment 2. Although the stoichiometry of this complex remains to be determined, the balance of the data so far indicates that the first explanation is correct.

We have interpreted the cooperativity of repressor binding in terms of protein-protein interactions; a second explanation, however, also seems possible. Binding of one repressor dimer could alter the conformation of the DNA site in such a way as to facilitate binding of adjoining repressors. In the perfect consensus site the centre of the operator sequence is —CTAG—. The finding that nucleotide steps of TA in oligonucleotide crystals and the *trp* repressor-operator complex¹⁸ induce DNA bending towards the major groove could support this argument. None of the naturally occurring *met* operators, however, contains the sequence 5'-CTAG-3'.

Comparison to *trp* repressor

Structural work most comparable to ours with an *E. coli* metabolic repressor has been with the *trp* repressor. This repressor is also a stable dimer in solution, having intertwined regions of polypeptide. It differs, however, from MetJ in that it has a 'classical' helix-turn-helix DNA-binding motif, and also in that the binding of corepressor results in a large coordinate conformational change around this DNA-binding region^{9,19}. Analysis of the natural *trp* operators does not immediately reveal obvious tandem repetitions. But recently, Gunsalus *et al.*^{20,21} proposed tandem-repressor-binding for the *trp* repressor on the basis of methylation- and nuclease-protection data, although the possibility of cooperativity in terms of protein-protein interaction was not discussed. Footprinting data for the *trp* repressor on natural operators indicate that more than one repressor must be bound^{14,21,22} and there is a report of an 8-bp repeat in the bound complex²³.

Remarkably, the *trp* operators contain as consensus elements a sequence that matches the met-box consensus sequence, that is, the repeat dAGNNNCT (N is an unspecified nucleoside). Indeed, it is possible to analyse the naturally occurring *trp*-operator regions in terms of 'trp boxes' analogous to met boxes, that is, as several 8-bp repeats with similar dyad symmetry. This raises the possibility that *met* and *trp* operators have evolved from a common ancestor, even though their respective repressor proteins are structurally unrelated and operate differently. In fact, Sigler *et al.*¹⁸ report that the structure of the *trp* repressor complexed with a symmetrized DNA fragment that contains a portion of a natural operator site is not inconsistent with a similar model for tandem binding.

The tandem-binding models discussed here may be of general relevance for other gene regulatory proteins that seem to recognize tandem sites that do not contain 2-fold symmetry. □

Note added in proof: See note added in proof on page 710 of this issue³.

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1. Ptashne, M. *A Genetic Switch* (Cell, California & Blackwell, Palo Alto, 1986).
2. Ptashne, M. *Nature* **335**, 683-689 (1988).
3. Rafferty, J. B., Somers, W. S., Saint-Girons, I. & Phillips, S. E. V. *Nature* **341**, 705-710 (1989).
4. Saint-Girons, I., Parsot, C., Zakim, M. M., Bärzu, O. & Cohen, G. N. *CRC Critical Reviews in Biochemistry* Vol. 23 S1-S42. (CRC, Cleveland, 1988).
5. Saint-Girons, I. *et al. J. biol. Chem.* **261**, 10936-10940 (1986).
6. Shoeman, R. *et al. Proc. natn. Acad. Sci. U.S.A.* **82**, 3601-3605 (1985).
7. Belfaiz, J. *et al. Proc. natn. Acad. Sci. U.S.A.* **83**, 867-871 (1986).
8. Plamann, L. S. & Stauffer, G. V. *J. Bact.* **169**, 3932-3937 (1987).
9. Zhang, R. G. *et al. Nature* **327**, 591-597 (1987).
10. Bae, S.-J., Chou, W.-Y., Matthews, K. & Sturtevant, J. M. *Proc. natn. Acad. Sci. U.S.A.* **85**, 6104-6108 (1988).
11. Galas, D. J. & Schmitz, A. *Nucleic Acid Res.* **5**, 3157-3170 (1978).
12. Siebenlist, U. & Gilbert, W. *Proc. natn. Acad. Sci. U.S.A.* **77**, 122-126 (1980).
13. Fried, M. & Crothers, D. M. *Nucleic Acids Res.* **9**, 6505-6525 (1981).
14. Carey, J. *Proc. natn. Acad. Sci. U.S.A.* **85**, 975-979 (1988).

15. Davidson, B.-E. & Saint-Girons, I. *Molec. Microbiol.* (in the press).
16. Hertzberg, R. P. & Dervan, P. B. *J. Am. chem. Soc.* **104**, 313-315 (1982).
17. Jordan, S. R. & Pabo, C. O. *Science* **242**, 893-899 (1988).
18. Otwinowski, Z. *et al. Nature* **335**, 321-329 (1988).
19. Schevitz, R. W., Otwinowski, Z., Joachimiak, A., Lawson, C. L. & Sigler, P. B. *Nature* **317**, 782-786 (1985).
20. Kumamoto, A. A., Miller, W. G. & Gunsalus, R. P. *Genes Dev.* **1**, 556-564 (1987).
21. Bass, S., Sugiono, P., Arvidson, D. N., Gunsalus, R. P. & Youderian, P. *Genes Dev.* **1**, 565-572 (1987).
22. Oppenheim, D. S., Bennett, G. N. & Yanofsky, C. *J. molec. Biol.* **144**, 133-142 (1980).
23. Chen, C.-H.B. & Sigman, D. S. *Science* **237**, 1197-1201 (1987).
24. Maxon, M. E. *et al. Proc. natn. Acad. Sci. U.S.A.* **86**, 85-89 (1989).
25. Riggs, A. D., Suzuki, H. & Bourgeois, S. *J. molec. Biol.* **48**, 67-83 (1970).
26. De Reuse, H., Tovati, E., Glaser, P. & Dauchin, A. *FEMS Microbiol. Lett.* **37**, 193-197 (1986).

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Muscle-specific gene expression controlled by a regulatory element lacking a MyoD1-binding site

T. J. Baldwin & S. J. Burden

Biology Department, 16-820 Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA

Muscle-specific expression of the gene encoding the delta subunit of the acetylcholine receptor is controlled by a 54-base-pair region that does not contain a binding site for MyoD1, a protein involved in activation of the myogenic program. A MyoD1-binding site is present in the proximal promoter region of the gene encoding the δ -subunit, but is neither sufficient nor necessary for muscle-specific expression in transfected muscle cells.

THE analysis of the regulation of skeletal muscle genes provides a particularly useful system in understanding mechanisms that are responsible for cell-type-specific gene expression. In particular, identification of MyoD1 and myogenin, gene products that are capable of altering the fate of mesodermal cells and activating the myogenic phenotype, provides one of the clearest examples in vertebrates of genes that are critical for early decisions in cell determination and differentiation¹⁻³. Although it is not clear how expression of MyoD1 and myogenin results in subsequent activation of genes that are specifically expressed in skeletal muscle cells, it has been suggested that MyoD1 may interact directly with these genes and activate transcription¹.

We have characterized the regulatory region of the murine acetylcholine receptor (AChR) δ -subunit gene by transfection assays and identified a 54-base pair (bp) region of 5' flanking DNA that is necessary and sufficient for muscle-specific gene expression. In addition, we identify a MyoD1-binding site in the δ -subunit gene. This MyoD1-binding site is not in the 54-bp region and not required for muscle-specific gene expression.

We demonstrate that nuclear proteins from myotubes, but neither myoblasts nor fibroblasts, bind to the 54-bp regulatory region and that these myotube-specific DNA-binding activities do not contain MyoD1. These studies demonstrate that DNA-binding activities, which are distinct from MyoD1, interact with a regulatory element that controls muscle-specific expression, and raise the possibility that these activities are critical for muscle-specific gene expression. In addition, we discuss possible roles for the MyoD1-binding site in the δ -subunit gene that are distinct from simple transcriptional activation.

Cis-acting regulatory element

Deletions of the δ -subunit gene were analysed to identify sequences that are necessary for muscle-specific expression. These constructs were transfected into C2 myoblasts or 3T3 cells and expression analysed in C2 myoblasts, C2 myotubes that had differentiated from the transfected C2 myoblasts, or in 3T3 cells. We demonstrated previously that nucleotides -148 to +24 of the δ -subunit gene are sufficient to confer cell-type and differentiation-dependent expression⁴. Deletion from nucleotide -148 to nucleotide -128 resulted in a 16-fold decrease in expression and deletion to nucleotide -107 resulted in a further 3-fold decrease (Fig. 1a; Table 1). Expression from nucleotides -148 to +24 was ~100-fold greater in C2 myotubes than 3T3 cells, whereas expression from nucleotides -107 to +24 was

equivalent in the two cell types (Table 1). Nucleotides -148 to -108 are therefore necessary for myotube-specific gene expression.

To determine whether the region identified as necessary for muscle-specific expression is also sufficient, we constructed chimaeric promoters containing regions of the δ -subunit gene fused to the *c-fos* basal promoter (FBP). This promoter contains a TATA element, a transcription start site, but lacks the serum response element⁵ and is an equally poor promoter in fibroblasts, myoblasts and myotubes (Fig. 1b; Table 1).

Fusion of nucleotides -148 to -53 from the δ -subunit gene immediately 5' to the FBP conferred myotube-specific expression on the FBP (Fig. 1b; Table 1). Deletion of nucleotides

TABLE 1 Nucleotides -148 to -95 are necessary and sufficient to confer myotube-specific expression

Construction	CAT activity (%)		
	Myotubes	Myoblasts	3T3
148/+24	100.0±12.0	4.4±0.7	0.4±0.2
-128/+24	6.3±1.8	3.0±0.6	4.0±0.7
-107/+24	2.0±0.8	0.8±0.2	2.0±0.2
SP65	0.5±0.2	0.2±0.2	0.2±0.2
SV40	125.0±17.0	88.0±11.0	96.0±12.0
-148/-140-FBP	3.2±1.1	0.9±0.2	0.8±0.4
-140/-148-FBP	3.7±1.2	0.8±0.3	0.9±0.6
-148/-128-FBP	3.0±0.9	1.1±0.2	0.9±0.7
-128/-148-FBP	3.4±1.3	0.7±0.2	0.8±0.2
-150/-107-FBP	3.3±1.7	0.8±0.2	1.0±0.3
-107/-150-FBP	3.7±1.2	0.9±0.3	0.8±0.2
-148/-95-FBP	66.0±8.0	2.4±1.1	1.0±0.2
-95/-148-FBP	59.0±14	2.0±1.4	1.1±0.4
-148/-53-FBP	68.0±17	2.7±1.6	1.0±0.2
-53/-148-FBP	66.0±12	3.0±1.2	1.2±0.3
FBP	1.8±0.9	0.7±0.6	1.1±0.2
FBP, s.s.	1.3±0.7	0.8±0.4	0.9±0.2
-107/+24-CAT	1.7±0.7	ND	0.6±0.3
-107/+24-CAT-(-95/-148)	1.1±0.8	ND	0.5±0.3
-107/+24-CAT-(-95/-148) ₃	3.8±1.1	ND	ND
-107/+24-CAT-(-53/-148)	1.3±0.9	ND	0.8±0.5
-107/+24-CAT-SVE	48.0±9.0	ND	38.0±11.0

(n=4-6)

The activity of chloramphenicol acetyl transferase (CAT) in cultures transfected with nucleotides -148 to +24 of the δ -subunit gene was arbitrarily assigned as 100% and corresponds to 86% conversion of labelled chloramphenicol to its acetylated derivatives. The mean activity and the standard error of the mean from four to six separate assays are presented. The construct -150/-107-FBP was made by cloning a double-stranded oligodeoxynucleotide extending from nucleotides -150 to -107 of the δ -subunit gene into the FBP-CAT vector described in the legend to Fig. 1. The sequence of this construct was verified by direct DNA sequencing¹⁸. Cells transfected with FBP and stimulated with serum are referred to as FBP, s.s.¹⁹. There is a higher level of expression from the δ -subunit gene promoter in myoblasts than 3T3 cells, which is probably because of incipient differentiation of myoblasts. Nevertheless, deletion of nucleotides -148 to -129 results in a small increase of expression in 3T3 cells, but not in myoblasts. We have not investigated whether this increase in expression in 3T3 cells arises from removal of sequences involved in repression in 3T3 cells or from inadvertently placing activating plasmid sequences in proximity to the δ -subunit gene basal promoter. Nevertheless, the maximal level of expression in 3T3 cells transfected with nucleotides -128 to +24 is 25-fold lower than expression in myotubes transfected with nucleotides -148 to +24. The orientation of DNA from the δ -subunit gene that was placed 3' to the CAT-encoding sequence was determined by DNA sequencing¹³. The orientation of the three copy insert of nucleotides -148 to -95 is -148 to -95, -148 to -95 and -95 to -148. The SV40 enhancer (SVE)^{10,20} is oriented 3'-to-5' as determined by mapping with *Nco*I. ND, not determined.

–96 to –53 of the δ -subunit gene did not diminish expression significantly, whereas further deletion to nucleotide –107 resulted in a 30-fold decrease in expression (Fig. 1b; Table 1). Nucleotides –148 to –95 are sufficient, and nucleotides –106 to –95 are necessary, to confer myotube-specific expression.

Nucleotides –148 to –95 activated transcription equally well in either orientation when positioned immediately 5' of the FBP (Table 1). Although we have not investigated the position-dependency in detail, neither single nor multiple copies of the 54-bp element activated expression when positioned ~1.7 kilobases (kb) 3' to the δ -subunit basal promoter (Table 1). The δ -subunit basal promoter retained functional promoter capabilities, however, because addition of the SV40 enhancer activated transcription 30-fold from the basal promoter of the δ -subunit gene in fibroblasts and myotubes (Table 1).

Sequence motif in the 54-bp element

Because expression studies demonstrate that both nucleotides –148 to –129 and –106 to –95 are necessary for myotube-specific expression (Table 1) and binding studies demonstrate that myotube nuclear proteins bind to both the 5' and 3' region of the –148 to –95 sequence (see below; Fig. 2), we examined the DNA sequence of the 54-bp element to determine whether a similar motif is present both in 5' and 3' portions of the –148 to –95 sequence. Indeed, an imperfect repeat of seven nucleotides, TGCCTGG, occurs four times in the 54-bp element (Table 2). A single copy of this sequence is present in the myotube-specific regulatory region of the gene for the chicken AChR α -subunit^{6,7} and two copies in the murine creatine kinase gene⁸ (Table 2). Nevertheless, this motif is not apparent in the important regulatory region of the skeletal muscle actin gene⁹ or in the myotube-specific enhancer located 3' to the rat myosin light-chain 1/3 gene¹⁰. Furthermore, this motif alone is not sufficient to confer myotube-specific expression, because

mutated δ -subunit genes that maintain either one copy of the motif (construct –148/–140 FBP-CAT) or three copies of the motif (construct –128/+24-CAT) did not confer myotube-specific expression. Nevertheless, it is interesting that a single linker substitution in the murine creatine kinase gene that alters both the MyoD1-binding site and one of the motifs described above virtually abolishes myotube-specific expression⁸.

Binding of myotube nuclear protein to 54-bp element

We used an electrophoretic mobility shift assay (EMSA) to determine whether nuclear proteins expressed in myotubes bind to the 54-bp element. Nuclear proteins from C2 myotubes bound to nucleotides –148 to –95 and formed one major and three minor protein–DNA complexes (Fig. 2). Because competition with unlabelled –148 to –95 DNA inhibited the appearance of each shifted band, whereas competition with unlabelled plasmid DNA caused only a small reduction in protein–DNA complexes, each shifted band arises from a sequence-specific interaction of nuclear protein(s) with the 54-bp element. It is of interest that nuclear protein(s) from C2 myotubes bound to either nucleotides –148 to –129 or –129 to –95 (Fig. 2). Furthermore, because nucleotides –148 to –140 compete partially with nucleotides –148 to –95 for binding of nuclear proteins, at least part of the sequence that these nuclear proteins recognize is contained within the TGCCTGG motif.

Nuclear proteins isolated from undifferentiated C2 myoblasts and 3T3 cells also bound to the 54-bp element (Fig. 2). It is important to note that the electrophoretic mobility of the shifted-bands detected with extracts from differentiated myotubes was different from that detected with myoblast extracts. Furthermore, because nucleotides –129 to –95, but not nucleotides –148 to –129, compete efficiently for binding, nuclear proteins from myoblasts bind to the 3' region, but not the 5' region of the 54-bp element.

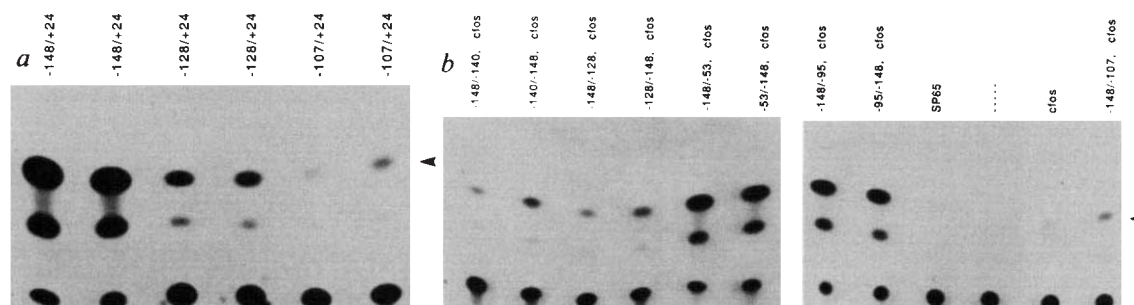


FIG. 1 Nucleotides –148 to –95 of the murine AChR δ -subunit gene are necessary and sufficient to confer myotube-specific expression. **a**, Autoradiogram of TLC fractionation of the reaction products in a CAT assay from transiently transfected C2 myotubes demonstrates that CAT activity is present in myotubes transfected with nucleotides –148 to +24 of the δ -subunit gene, but that 16-fold and 50-fold lower levels of CAT activity are present in myotubes transfected with nucleotides –128 to +24 and –107 to +24 of the gene, respectively. The results from duplicate transfections are illustrated. **b**, Autoradiogram of TLC fractionation of the reaction products in a CAT assay from transiently transfected C2 myotubes demonstrates that CAT activity is present in myotubes transfected with chimaeric promoters containing either nucleotides –148 to –53 or –148 to –95 of the δ -subunit gene in either orientation, immediately 5' to the *c-fos* basal promoter (FBP). Transcription from the chimaeric promoter is also accurate, because transcription from the normal *c-fos* gene and the chimaeric promoter initiate at the same nucleotide (unpublished results). CAT expression from the FBP is low (*cfos*), but greater than the level of CAT activity observed in non-transfected myotubes (–) or myotubes transfected with a plasmid lacking a promoter (SP65). Slightly elevated levels of CAT activity are present in myotubes transfected with chimaeric promoters containing nucleotides –148 to –140, –148 to –128, and –148 to –107 upstream of FBP. The arrowheads indicate the position of 3-mono-acetylated chloramphenicol. The TLC plates were exposed to X-ray film at room temperature overnight.

METHODS. The methods used to generate 5' deletions of the δ -subunit gene with *Bal31* nuclease have been described previously⁴. Constructs containing 148, 128 or 107 bp of 5' flanking DNA also contain 24 bp of 5' untranslated region and were fused to the coding region of the CAT gene as described⁴. To construct the chimaeric promoters, an *EcoRI*–*HindIII* cassette containing δ -subunit-gene nucleotides –148 to +24 was cloned into *EcoRI* and *HindIII* digested pGEM-3. The DNA was linearized with *HindIII*, partially digested with *Bal31* and then digested with *EcoRI*. T4 DNA polymerase was used to produce blunt ends and *Sall* linkers were ligated to blunt-ended DNA. The 3' deleted DNAs were digested with *Sall*, purified and cloned into a plasmid containing a unique *Sall* site immediately 5' to the basal promoter of the murine *c-fos* gene containing nucleotides –56 to +109 from the *c-fos* gene⁵. Plasmids containing δ -subunit sequences 3' to the CAT gene were constructed by isolating δ -subunit sequences from the chimaeric promoters described above and cloning these *Sall* fragments into a unique *Sall* site 3' to the CAT coding sequence in the SP65(–107/+24)–CAT construct. DNA was transfected as a calcium phosphate precipitate^{4,21}. We co-transfected cells with a plasmid containing the human growth hormone (HGH) gene²² and normalized CAT expression to a constant level of secreted HGH⁴. HGH activity varied less than twofold between the two different cell lines, and there was less than two-fold variation in HGH activity in different experiments in any one cell line. The assays were quantitated by excising radioactive chloramphenicol and its acetylated derivatives from TLC plates and measuring the radioactivity by scintillation counting⁴.

TABLE 2 The TGCCTGG motif

Gene	Regulatory region	Motif
Chick AChR alpha subunit	-116 to -81	TGCCTGG -98 to -92
Mouse creatine kinase	-1224 to -1114	TGCCTGG -1173 to -1167
		TGCCTGA -1123 to -1117
Mouse AChR δ -subunit	-148 to -95	TGCCTGG -148 to -142
		TGCCCTTG -128 to -121
		TGCCCTAA -115 to -108
		TGGCAAAC -106 to -99

-150	-140	-130	-120	-110	-100	-95
^	^	^	^	^	^	^
CCTGCCTGGGATCTTTTCGTCTGCCCTTGCCCTCTGCCTAACTGGCAAACCCCA						
TGCCTGG		TGCCCTTG		TGCCCTAA	TGGCAAAC	

A motif is repeated in the myotube-specific regulatory region of the δ -subunit gene and present in the regulatory region of other genes expressed in skeletal muscle. Genes which contain TGCCTGG in their myotube-specific regulatory region are indicated⁶⁻⁸; the sequence of the motif and the position of the motif in each gene are indicated. The location of this motif in nucleotides -150 to -95 of the δ -subunit gene is shown.

MyoD1 does not bind to the 54-bp element

MyoD1, a nuclear protein that has sequence similarity with the *myc* family of DNA-binding proteins^{1,11}, is a skeletal muscle-specific gene product that can trigger the differentiation of 10T1/2 cells and alter the differentiation of fibroblast lines to a myogenic phenotype¹. We were interested in determining whether the δ -subunit gene contains a binding site for MyoD1 and, if so, whether the binding site is in the 54-bp myotube-specific regulatory region.

We identified such binding sites by EMSA. The MyoD1 protein used in these assays was a recombinant protein between MyoD1 and glutathione S-transferase (MyoD1-glu)¹². The MyoD1-glu protein binds to a DNA fragment extending from nucleotide -148 to +24 of the δ -subunit gene (Fig. 3). The binding of MyoD1-glu was competed efficiently with unlabelled -148 to +24 DNA and with DNA from the 3' region (nucleotides -95 to +24), but not the 5' region (nucleotides -148 to -95)

(Fig. 3). MyoD1 therefore binds to DNA sequences between nucleotides -95 to +24, but not to 54-bp element.

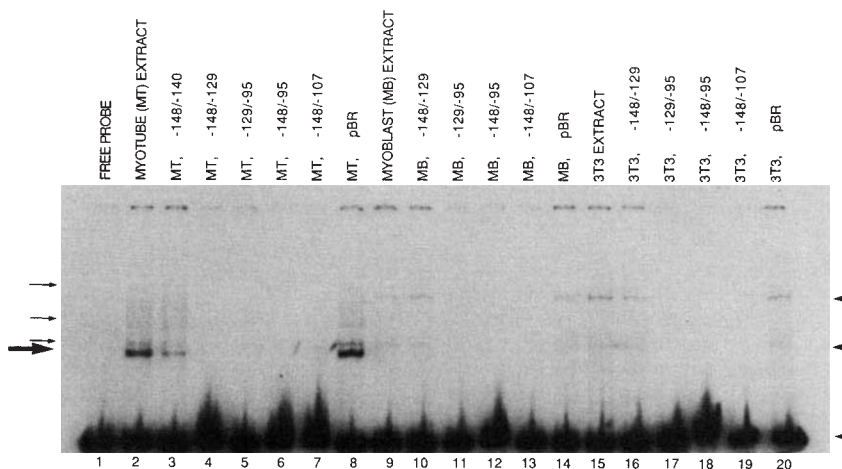
The MyoD1-binding site was mapped more precisely by methylation interference footprinting assays¹³. These studies demonstrate that methylation of guanosine residues -23, -22, -17 and -15 interfere with the binding of MyoD1, whereas methylation of guanosine residue -19 enhances MyoD1-binding (Fig. 4). The MyoD1-binding site in the δ -subunit gene therefore extends from nucleotides -23 to -15.

Expression and the MyoD1-binding site

The expression studies along with the binding studies that we have described demonstrate that a MyoD1-binding site is not sufficient to confer myotube-specific expression, because nucleotides -107 to +24 of the δ -subunit gene retain the MyoD1-binding site, yet do not confer myotube-specific expression. Nucleotides -107 to +24, however, retain functional promoter

FIG. 2 Nuclear proteins from C2 myotubes bind to nucleotides -148 to -95 in the δ -subunit gene. One major (thick arrow) and several minor shifted-bands (thin arrows) are detected in binding assays of nuclear proteins from C2 myotubes and nucleotides -148 to -95. Binding is competed partially with nucleotides -148 to -140 and completely with nucleotides -148 to -95, -148 to -107, -148 to -129 and -128 to -95. No competition is detected with 1 μ g *Msp*I-digested pBR322 DNA. Two minor shifted-bands (large arrowheads) are detected in binding assay of nuclear proteins from either C2 myoblasts or 3T3 cells and nucleotides -148 to -95. Binding is competed completely with nucleotides -148 to -95, -148 to -107, and -128 to -95. No competition is detected with nucleotides -148 to -140, -148 to -129 or pBR322 DNA. Three micrograms of nuclear protein was included in each assay and competition assays included a 100-fold molar excess of purified competitor DNA. The position of free DNA probe is indicated with a small arrowhead.

METHODS. Nuclear extracts from C2 myotubes, C2 myoblasts and NIH 3T3 cells were prepared essentially as previously described²³; the solubilization buffer, however, included leupeptin (10 μ g ml⁻¹) and aprotinin (10 μ g ml⁻¹). C2 myoblasts were collected at 20–30% confluence and NIH 3T3 cells at ~80% confluency. Differentiation of C2 myotubes was initiated by growing 70–80% confluent C2 myoblasts in 5% adult bovine serum for three days and subsequently in 5% adult bovine serum medium supplemented with cytosine arabinoside (10⁻⁵ M) for two days to minimize growth of myoblasts. Five days after induction of differentiation the C2 myotubes were collected. Labelled DNA fragments were prepared by digesting pGEM(-148/+24) DNA with *Eco*RI and end-labelling with [α -³²P]dATP (6,000 Ci mmol⁻¹)²⁴.



The DNA was subsequently digested with *Hind*III and labelled DNA (nucleotides -148 to +24) was isolated by PAGE. After digestion with *Hph*I and gel electrophoresis, labelled DNA (nucleotides -148 to -95) was isolated (10⁷–10⁸ c.p.m. μ g⁻¹) and used in EMSA. EMSA with nuclear extracts was performed in 10- μ l reactions containing 5,000 c.p.m. (0.1 ng) labelled DNA and 3 μ g nuclear extract protein (in 40 mM KCl, 2 mM MgCl₂, 1 mM EDTA, 8% glycerol, 10 mM Tris buffer, pH 7.4 and 1 μ g poly(dI-dC)·poly(dI-dC)²⁵⁻²⁷. The reactions were at 22 °C for 15 min. Free DNA and protein-DNA complexes were separated by PAGE (150 V; 4% acrylamide, 22 mM Tris buffer, 22 mM boric acid and 0.5 mM EDTA pH 8.0). DNA fragments used in competition assays were purified from plasmids containing the relevant fragment and were added to the labelled DNA fragment before addition of nuclear extract.

capabilities, as addition of the SV40 enhancer to a construct containing these nucleotide elevates expression in both muscle and fibroblasts (Table 1).

Similarly, a MyoD1-binding site is not necessary for muscle-specific expression in these transfection assays, because a chimaeric promoter between the 54-bp element and the FBP does not contain a MyoD1-binding site (Fig. 3b) yet confers muscle-specific expression (Table 1).

Discussion

We have demonstrated that a 54-bp region of 5'-flanking DNA from the gene for the δ -subunit of the murine AChR is necessary and sufficient for muscle-specific gene expression. Deletion of this sequence results in a 50-fold decrease in expression in myotubes, and fusion of this sequence immediately 5' to the *c-fos* basal promoter confers myotube-specific expression on an otherwise weak, non-tissue-specific promoter.

MyoD1 does not bind to the 54-bp region. Indeed, preliminary experiments indicate that the four binding activities that do bind to the 54-bp element do not contain MyoD1: antibodies against MyoD1 react with MyoD1-glu, but not with the activities in C2 nuclear extracts that bind to the 54-bp element.

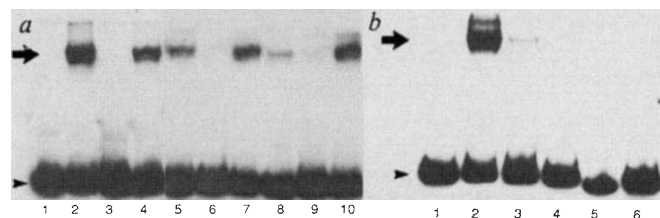


FIG. 3 MyoD1 binds to a region of the δ -subunit gene that is neither necessary nor sufficient for myotube-specific expression. *a*, MyoD1 binds to nucleotides -95 to +24, but not -148 to -95 of the δ -subunit gene. The probe is 32 P-end-labelled DNA (nucleotides -148 to +24) of the δ -subunit gene. The position of free DNA (lane 1) is indicated with an arrowhead. Incubation of labelled DNA with the MyoD1-glu recombinant protein results in a distinct shifted band (lane 2; arrow), whereas incubation with glutathione S-transferase does not result in a shifted band (lane 3). One hundredfold molar excess of unlabelled δ -subunit DNA (nucleotides -148 to -95) reduces binding by about twofold (lane 4); because pBR DNA also reduces binding by approximately twofold (lane 10), this competition is considered sequence nonspecific. By contrast, 10-fold (lane 5) and 100-fold (lane 6) molar excess of an oligodeoxynucleotide containing the murine creatine kinase MEF1-binding site compete for binding to the 32 P-end-labelled probe. Similarly, 10-fold (lane 8) and 100-fold (lane 9) molar excess of the δ -subunit DNA (nucleotides -95 to +24) compete for binding to 32 P-end-labelled probe. An equal molar quantity of δ -subunit DNA (nucleotides -95 to +24) (lane 7) and 1 μ g *Msp*I-digested pBR322 DNA (lane 10) reduce binding by approximately twofold. *b*, MyoD1 does not bind to the *c-fos* basal promoter (FBP). The DNA probe in lanes 1-4 is 32 P-end-labelled δ -subunit DNA (nucleotides -148 to +24) and in lanes 5 and 6 is 32 P-end-labelled *c-fos* DNA (nucleotides -56 to +109). The position of free DNA (lanes 1 and 5) is indicated with an arrowhead. Incubation of labelled δ -subunit DNA probe with MyoD1-glu results in a distinct shifted band (lane 2; arrow) that is competed with a 10-fold (lane 3) and 100-fold (lane 4) molar excess of unlabelled δ -subunit DNA (nucleotides -148 to +24). MyoD1 does not bind to FBP because incubation of labelled *c-fos* DNA probe with MyoD1-glu does not result in a shifted band (lane 6). The dried gel was exposed to X-ray film with an intensifying screen overnight at -70°C .

METHODS. pGEM-(-148/+24) DNA was labelled as described in Fig. 2. The double-stranded oligodeoxynucleotide representing the MEF1-binding site extends from nucleotides -1,138 to -1,117 in the murine creatine kinase gene⁸. EMSA with MyoD1-glu was performed in 20- μ l reactions containing 5,000 c.p.m. (0.1 ng) labelled DNA and 10 ng MyoD1-glu protein, or 10 ng of glutathione S-transferase (in 50 mM KCl, 3 mM MgCl_2 , 1 mM EDTA, 1 mM DTT, 0.5% Nonidet P-40 or NP-40, 10 μ g ml^{-1} BSA, 10% glycerol, 20 mM HEPES buffer, pH 7.6 and 5 μ g ml^{-1} of poly(dI-dC)·poly(dI-dC). Free DNA and protein-DNA complexes were separated by PAGE (6% acrylamide, 22 mM Tris buffer, 22 mM boric acid and 0.5 mM EDTA, pH 8.0). Fragments used in competition assays were purified from plasmids containing the relevant fragment and added to the labelled DNA fragment before addition of nuclear extract.

It is possible that myogenin, rather than MyoD1 is required for muscle-specific expression of the δ -subunit gene by binding to the 54-bp element. Consistent with this idea, the BC3H1 smooth-skeletal muscle cell line expresses AChR¹⁴ and myogenin², but not MyoD1¹. Nevertheless experiments indicate that nuclear extracts from C2 myotubes contain an activity that binds to the MyoD1-binding site (oligodeoxynucleotide of nucleotides -29 to -15) and that this activity reacts with

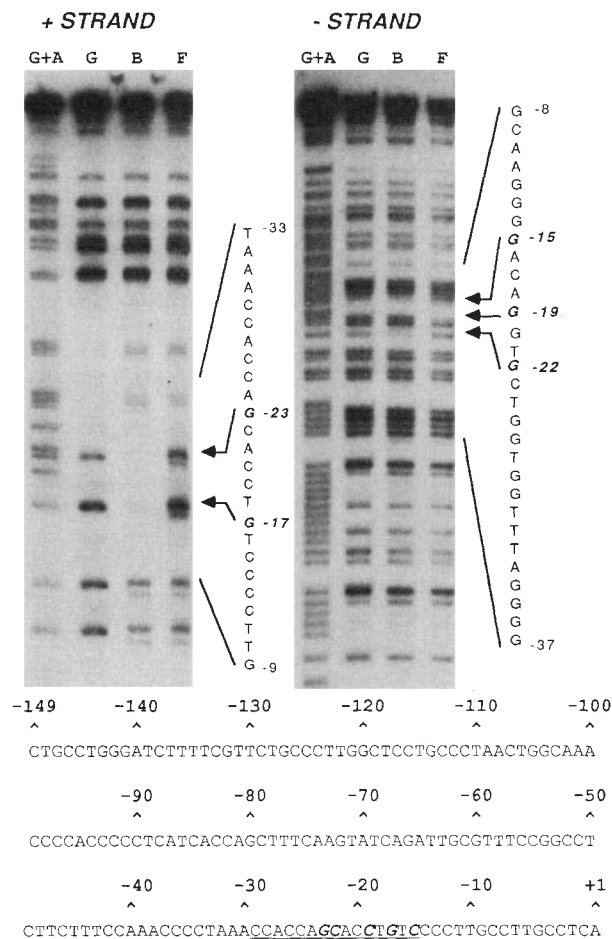


FIG. 4 Nucleotides -23 to -15 of the δ -subunit gene are involved in MyoD1 binding. Methylation of guanosine residues -17 and -23 on the (+) strand and -15 and -22 on the (-) strand interferes with MyoD1 binding, whereas methylation of guanosine residue -19 on the (-) strand enhances MyoD1 binding. The sequence of the δ -subunit gene from nucleotides -149 to +1 is illustrated. Residues identified by methylation interference are in bold and italics; the position of the methylated nucleotide that enhances MyoD1 binding is indicated with an asterisk. Ethylation of residues -29 to -15 (underlined) interferes with MyoD1 binding (unpublished observations). Products of Maxam-Gilbert sequencing reactions with free DNA are denoted by G+A and G. B and F are products of Maxam-Gilbert G-specific sequencing reactions with partially methylated DNA either complexed with MyoD1 (B) or free (F).

METHODS. The probe used for methylation interference footprinting contained sequences -107 to +24 of the δ -subunit promoter. The upper strand was labelled by digesting SP65-(-107/+24)-CAT DNA with *Hind*III and labelling the ends with [α - 32 P]dATP; after digestion with *Eco*RI, the labelled fragment from the δ -subunit gene was purified from polyacrylamide gels. The lower strand was labelled in a similar manner except that the DNA was digested first with *Eco*RI and subsequently with *Hind*III. End-labelled DNA was methylated with dimethylsulphate as described¹³. Partially methylated DNA (5×10^6 c.p.m.) was included in an EMSA containing 0.5 μ g MyoD1-glu and the bound and free DNA were excised from 4% polyacrylamide gels and isolated after chromatography on Elutip columns (Schleicher and Schuell, Keene, New Haven). The purified DNA was cleaved with piperidine¹³ and the products resolved in an 8% polyacrylamide-7M urea gel. The gels were fixed, dried and exposed overnight to X-ray film at -70°C with an intensifying screen.

antibodies to myogenin; furthermore, these antibodies do not react with the activities that bind to the 54-bp element (T.J.B., S.J.B. and Wright, W. E., unpublished results). Moreover, the 54-bp element does not compete with the MyoD1-binding site in binding assays. Neither MyoD1 nor myogenin alone can thus account for muscle-specific expression conferred by the 54-bp element.

Our MyoD1-binding assays may not have reflected the *in vivo* assembly state of MyoD1. Indeed, recent studies demonstrate that MyoD1 can form a heterodimer with either immunoglobulin-enhancer binding proteins E12 or E47 *in vitro*; importantly, these heterodimers bind a sequence similar to the MyoD1 binding site defined in our study with recombinant MyoD1-glu¹⁵. Independent, therefore, of whether MyoD1, myogenin or heterodimers of MyoD1 or myogenin complexed with more general transcription factors bind to this site *in vivo*, we conclude that this site is neither required nor sufficient for muscle-specific expression in these transfection experiments.

What is the role of the MyoD1-binding site in the δ -subunit gene and in other genes that are expressed in skeletal muscle? A MyoD1-binding site may be dispensable in the δ -subunit gene

and have a direct role in regulating expression of other genes expressed in skeletal muscle. In this regard, it is noteworthy that fusion-arrested myoblasts express AChR but do not express a variety of proteins associated with the contractile apparatus^{16,17}. Furthermore, C2 myoblasts transformed with activated *ras* or *fos* express little or no MyoD1, myogenin, myosin light chain and creatine kinase, but express reduced levels of desmin and nearly normal levels of AChR alpha subunit²⁸. Expression of some skeletal muscle genes could thus be regulated by a MyoD1-myogenin-dependent pathway, whereas other genes could be regulated by a separate pathway and involve activities that bind to the 54-bp element. By contrast, the MyoD1-binding site in the δ -subunit gene may have a role that was not detected in our assays. The MyoD1-binding site may play a part earlier in muscle development and is not detected in transfection assays of myoblasts and myotubes. Analysis of stably transfected cell lines or transgenic mice may distinguish among some of these possible functions of a MyoD1-binding site. Nevertheless, the results presented here indicate that a MyoD1-binding site in the δ -subunit gene has a function that is distinct from simple transcriptional activation. □

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1. Davis, R. L., Weintraub, H. & Lassar, A. B. *Cell* **51**, 987–1000 (1987).
2. Wright, W. E., Sassoon, D. A. & Lin, V. K. *Cell* **56**, 607–617 (1989).
3. Edmondson, D. & Olson, E. N. *Genes Dev.* **3**, 628–640 (1989).
4. Baldwin, T. J. & Burden, S. J. *J. Cell Biol.* **107**, 2271–2279 (1988).
5. Gilman, M. Z., Wilson, R. N. & Weintraub, H. A. *Molec. cell. Biol.* **6**, 4305–4316 (1986).
6. Wang, Y., Xu, H. P., Wang, X. M., Ballivet, M. & Schmidt, J. *Neuron* **1**, 527–534 (1988).
7. Piette, J., Klarsfeld, A. & Changeux, J. P. *EMBO J.* **8**, 687–694 (1989).
8. Buskin, J. N. & Hauschka, S. D. *Molec. cell. Biol.* **9**, 2627–2640 (1989).
9. Bergsma, D. J., Grichnik, J. M., Gossett, L. M. & Schwartz, R. J. *Molec. cell. Biol.* **6**, 2462–2475 (1986).
10. Donoghue, M., Ernst, H., Wentworth, B., Nadal-Ginard, B. & Rosenthal, N. *Genes Dev.* **2**, 1779–1790 (1988).
11. Tapscott, S. J., Davis, R. L., Thayer, M. J., Cheng, P. F., Weintraub, H. & Lassar, A. B. *Science* **242**, 405–411 (1988).
12. Lassar, A. B. *et al. Cell* **58**, 823–831 (1989).
13. Maxam, A. & Gilbert, W. *Meth. Enzym.* **65**, 499–560 (1980).
14. Boulter, J. & Patrick, J. *Biochemistry* **16**, 4900–4908 (1977).
15. Murre, C. *et al. Cell* **58**, 537–544 (1989).
16. Shainberg, A., Yagil, G. & Yaffe, D. *Dev. Biol.* **25**, 1–29 (1971).
17. Patterson, B. & Prives, J. *J. Cell Biol.* **59**, 241–245 (1973).
18. Chen, E. Y. & Seeburg, P. *DNA* **4**, 165–170 (1985).

19. Treisman, R. *Cell* **42**, 889–902 (1985).
20. Gruss, P., Dhar, R. & Khoury, G. *Proc. natn. Acad. Sci. U.S.A.* **78**, 943–947 (1981).
21. Wigler, M. *et al. Proc. natn. Acad. Sci. U.S.A.* **76**, 1373–1376 (1979).
22. Selden, R. F., Howie, K. B., Rowe, M. E., Goodman, H. M. & Moore, D. D. *Molec. cell. Biol.* **6**, 3173–3179 (1986).
23. Dignam, J. D., Liebowitz, R. D. & Roeder, R. H. *Nucleic Acids Res.* **11**, 1475–1489 (1983).
24. Maniatis, T., Fritsch, E. F. & Sambrook, J. *Molecular cloning* (Cold Spring Harbor Laboratory, New York, 1982).
25. Fried, M. & Crothers, D. M. *Nucleic Acids Res.* **9**, 6505–6525 (1981).
26. Strauss, F. & Varshavsky, A. *Cell* **37**, 889–901 (1984).
27. Singh, H., Sen, R., Baltimore, D. & Sharp, P. A. *Nature* **319**, 154–158 (1986).
28. Lassar, A. B. *et al. Cell* **58**, 659–667 (1989).

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LETTERS TO NATURE

Discovery of intergalactic radio emission in the Coma–A1367 supercluster

K.-T. Kim*, P. P. Kronberg*, G. Giovannini†‡ & T. Venturi‡

* Department of Astronomy, University of Toronto, Toronto, Ontario, Canada, M5S 1A7

† Dipartimento di Astronomia, via Zamboni 33, I-40100 Bologna, Italy

‡ Istituto di Radioastronomia, via Irnerio 46, I-40126 Bologna, Italy

THE Coma cluster is a rich cluster of galaxies nested in an even larger supercluster of galaxies. The plane of the supercluster seems to be defined by the Coma cluster itself and another galaxy cluster, Abell 1367, which lies ~40 Mpc farther west^{1,2}. The largest structures known are the giant voids and superclusters which are as large as $70h_{-75}^{-1}$ Mpc (refs 3–5). The Coma cluster of galaxies seems to be located on the rim of a giant void⁶ in the three-dimensional distribution of galaxies. Here we describe the detection of faint, supercluster-scale radio emission at 326 MHz that extends between the Coma cluster of galaxies (Abell 1656) and the Abell 1367 cluster and which is apparently not associated with any individual galaxy system in the complex. The radiation's synchrotron origin implies the existence of a large-scale intercluster magnetic field

with an estimated strength of 0.3–0.6 μ G, which is remarkably strong. The synchrotron-emitting relativistic electrons cannot be older than a few times 10^8 yr, but we speculate that the magnetic field is the fossil of a pre-galactic primeval field, which was amplified in the course of the formation of intergalactic voids and superclusters.

The Coma cluster of galaxies is visible at many wavelengths, from low radio frequencies to X-rays. It is thus an important testbed for large-scale phenomena in extragalactic astronomy. At radio frequencies, Coma is one of a few rich clusters that are known to have a diffuse halo. It has a steep radio spectrum, which is associated with non-thermal radio radiation from a population of 'old' relativistic electrons that are accelerated by a cluster-scale magnetic field^{7–13}.

We observed a portion of the Coma supercluster plane for a total of 72 h in May and June 1986 at 326 MHz, using the Westerbork Synthesis Radio Telescope (WSRT); for a description of the observing procedure see ref. 17. The observations were made in the redundancy mode¹⁴ to increase the dynamic range. The data reduction was performed using the DWARE, NRAO-AIPS and CLEAN software packages. The synthesized beam of the final map was 2×1 arcmin elongated along the north–south axis and the r.m.s. noise level of the map is 1.1 mJy per beam. The useful field of view covers an area of 2.6° radius around the field centre.

We used further very-large-array observations at 327 MHz in the higher resolution A and B configurations centred both on

TABLE 1 List of sources located on the 'bridge'

Name*	Right ascension (1950)	Declination (1950)	Flux density at 1 GHz† (mJy)	Spectral index	Note‡
1254 + 27W2	12 ^h 54 ^m 05.41 ± 0.01 ^s	27° 17' 22.2 ± 0.2"	156.4 ± 5.2	-0.39 ± 0.04	5C4.40
1254 + 27W3	12 ^h 54 ^m 12.98 ± 0.17 ^s	27° 45' 17.8 ± 3.5"	14.0 ± 2.0		§
1254 + 27W4	12 ^h 54 ^m 17.81 ± 0.02 ^s	27° 27' 10.9 ± 0.3"	67.5 ± 5.4	-0.91 ± 0.09	5C4.43
1254 + 27W5	12 ^h 54 ^m 24.15 ± 0.02 ^s	27° 20' 52.9 ± 0.3"	24.2 ± 2.0	-0.50 ± 0.09	
1254 + 27W6	12 ^h 54 ^m 25.46 ± 0.13 ^s	27° 37' 51.8 ± 2.2"	15.1 ± 0.7	-0.95 ± 0.11	
1254 + 27W7	12 ^h 54 ^m 32.62 ± 0.08 ^s	27° 20' 14.9 ± 1.0"	13.9 ± 1.5	-1.00 ± 0.25	
1254 + 27W8	12 ^h 54 ^m 54.67 ± 0.09 ^s	27° 30' 05.9 ± 1.1"	25.3 ± 1.1	-0.60 ± 0.10	5C4.50
1254 + 27W1	12 ^h 54 ^m 59.19 ± 0.10 ^s	27° 46' 05.5 ± 1.1"	91.5 ± 11.7	-1.19 ± 0.16	5C4.51
1255 + 27W1	12 ^h 55 ^m 29.91 ± 0.19 ^s	27° 53' 18.5 ± 2.2"	6.2 ± 0.9	+0.12 ± 0.37	
1255 + 27W3	12 ^h 55 ^m 34.19 ± 0.58 ^s	27° 46' 24.4 ± 4.8"	10.7 ± 1.2	+0.13 ± 0.27	
1255 + 27W2a	12 ^h 55 ^m 47.00 ± 0.03 ^s	27° 50' 57.8 ± 0.3"	27.7 ± 1.8	-0.46 ± 0.08	5C4.61

* WSRT designation¹¹ of the source.

† K.-T. K., P. P. K., P. E. Dewdney and T. L. Landecker, manuscript in preparation.

‡ 5C designations⁹.

§ 326-MHz flux density (mJy), (G. G., unpublished data).

the cluster centre and on 1253 + 275, to find and subtract discrete sources present in the 'bridge' region. In particular, we have carefully subtracted the extended radio galaxies NGC4839 and NGC4827. The residuals are very small and we can rule out the possibility that the 'bridge' region is an artefact of the low-order interferometer spacings.

After removal of the small-diameter sources in this area, the remaining data were convolved to obtain a lower resolution (4×2 arcmin, elongated N-S) but higher signal-to-noise ratio for the diffuse emission. The resulting map (Fig. 1) has a dynamic range of $\sim 2,200:1$. The size of the 'bridge' is estimated to be $\sim 50 \times 20$ arcmin ($\sim 1.5h_{75}^{-1}$ Mpc in projection, where h_{75} denotes the use of $75 \text{ Mpc km}^{-1} \text{ s}^{-1}$ for the Hubble constant) and it is oriented along the axis defined by position angle about 50° (measured eastward from north). The total flux density S contained in the region is 760 ± 100 mJy at 326 MHz. Table 1 contains a list of sources seen on or near the 'bridge'.

The large extent and especially the radio morphology of the 'bridge' implies that it is not the combination of numerous unresolved weak radio sources located in the same region, but that it is related to the extension of the cluster and, we suggest, a feature of the Coma-A1367 supercluster. The ability to distinguish such extended synchrotron emission is due to a combination of the high sensitivity and resolution at low frequencies of the recently extended WSRT. The resolution is comparable to a galaxy size at the distance of Coma and enables us to distinguish individual radio galaxies from the extended emission.

The shortest interferometer spacing D of the WSRT is 36 m. The missing interferometer spacings, < 36 m, would produce a

negative 'bowl' of a diameter of about λ/D , if there is such a large-scale radio feature present in the sky. At 326 MHz, this corresponds to a gaussian source with half-power beamwidth of $\sim 1.5^\circ$. Because the total extent of the diffuse emission in the map is comparable to the scale of the negative 'bowl', it is thus possible that some weak extended features could have remained undetected. To test for this, we measured the zero-level of the map at different regions well away from the 'bridge' region. Although it is very close to zero in the peripheral regions of the map, it becomes negative (down to -2 mJy) in the central region. This implies that some amount of flux was indeed missed because of the lack of short-spacing data in the observations. We estimate the total missing flux density in Fig. 1 to be 100 mJy; its presence would further reinforce the 'bridge' emission. No polarization information is available from the present data.

The 'bridge' emission has now been independently verified *a posteriori* in two previous maps of the Coma-A1367 supercluster region—the recent DRAO (Dominion Radio Astrophysical Observatory) data¹³ at 408 MHz and the 430-MHz Aricebo telescope map⁹.

We have reanalysed the former observations, made in April and May 1985 with a field centre at RA $12^{\text{h}}55^{\text{m}}30^{\text{s}}$, dec $+27^\circ55'$. The sensitivity was 8 mJy per beam and the resolution at 408 MHz was 7.45×3.45 arcmin (elongated along the N-S axis.) Unlike the 326-MHz observations, single-dish data were available at 408 MHz from the Effelsberg Telescope¹⁵, and these were added to the DRAO observations. Thus, this combined observation is complete to the longest spacing of 600 m. (K.-T. K., P. P. K., P. E. Dewdney and T. L. Landecker, manuscript in

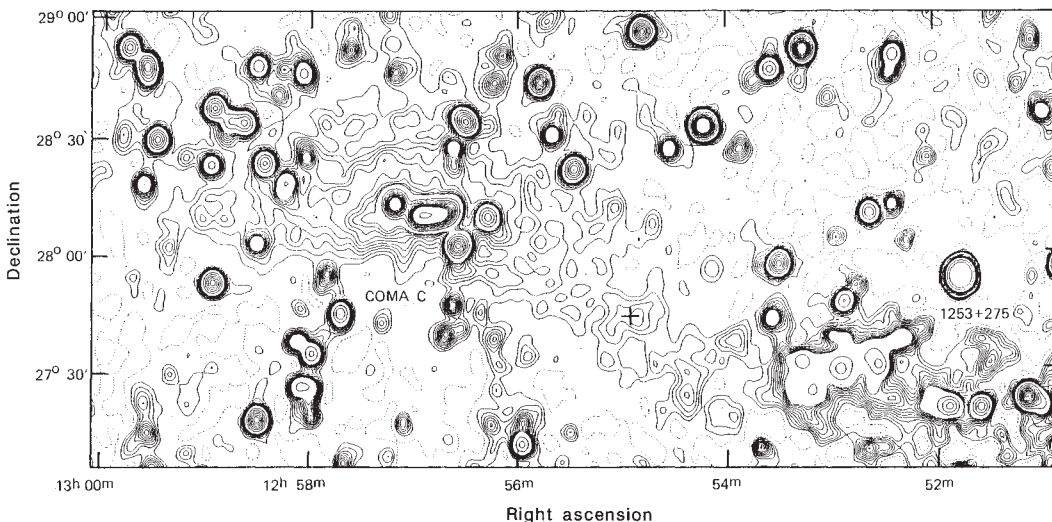
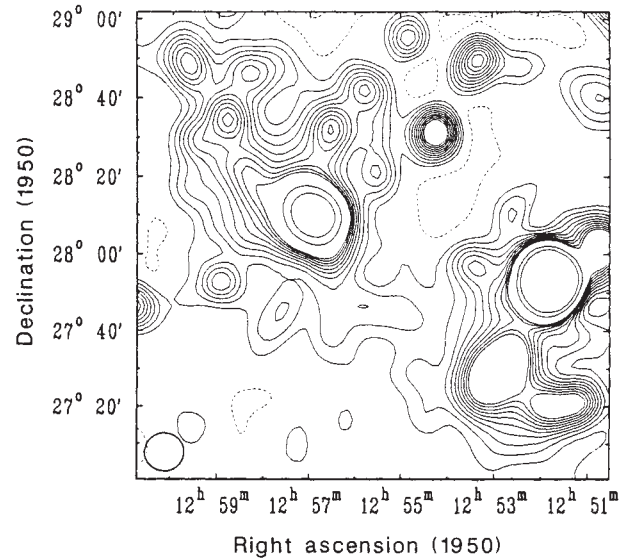


FIG. 1 The Coma cluster of galaxies was observed with the WSRT at 326 MHz. Ten antennas were fixed, and the four moveable ones were set at $B_n + n72$ m where $n=0$ to 37, and B_n (the shortest baselines) were 35, 48, 60, 72, 84 and 96 for six separate 12-h sessions. The projected linear size of this source is $\sim 1.5h_{75}^{-1}$ Mpc. The location of NGC4839 is marked with a + symbol. The peak surface brightness is 9.1 mJy per beam. Contours are shown at $-1, 1, 2, \dots, 9, 10, 20, 30, \dots, 90, 100, 200, 400$ times 4 mJy per beam.

FIG. 2 The DRAO 408-MHz map of the Coma cluster, using data which includes the single-dish observations. The synthesized beam is shown on the lower-left corner of the map. Contour levels are $40 \times (-1, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30)$ mJy per beam. Relatively strong small-diameter sources (1254+27W4, 1254+27W5, 1254+27W8, 1254+27W1 and a complex of sources to the south-east of Coma C) have been removed from this map.



preparation).

Small-diameter sources, the position and fluxes of which are well determined from the WSRT map, were removed from the 'bridge' region, and the resulting map was convolved (final resolution 10 arcmin) to enhance the signal-to-noise ratio. To minimize the source-subtraction errors, only reasonably strong sources were removed from the DRAO map. They are 1254+27W4 (5C4.43), 1254+27W5, 1254+27W8 (5C4.50), 1254+27W1 (5C4.51) (see Table 1), in addition to a complex of sources to the south-east of Coma C. The resulting map is shown in Fig. 2. We estimate the 408-MHz bridge flux to be 600 ± 100 mJy.

Evidence for the 'bridge' emission is also suggested in the 430-MHz Arecibo telescope map⁹, but the relatively low resolution and less certain beam and pointing characteristics did not make it possible to unambiguously ascribe the feature in that map to diffuse intergalactic emission. At 430 MHz, the total flux measured from the Arecibo telescope map is 600 ± 100 mJy. This is consistent with our estimates above (assuming a spectral index $\alpha = -1.5$), when we include the flux density missing from the 326-MHz WSRT map.

The physical parameters of the 'bridge' region can be estimated using the observed flux density and size at 326 MHz, $\alpha = -1.5$ ($S_\nu \propto \nu^\alpha$ where S_ν is the flux density at frequency ν) and the assumption of equipartition between the energy density of the relativistic protons and electrons and the magnetic field. If we also assume a volume-filling factor, ϕ , of unity, and a proton/electron ratio, k , of 1, the equipartition field strength is $0.3 \mu\text{G}$ if we model the 'bridge' as a cylinder 1,900 kpc long (allowing for some de-projection) and 800 kpc in diameter, and has a corresponding total energy of 1.4×10^{49} erg.

The 'dense' plane of the Coma supercluster may alternatively be a projection of a sheet-like boundary between the bubble surfaces around two spherical intergalactic voids⁶. In this case the magnetized region giving rise to the 'bridge' in Fig. 1 could have considerable depth d , along our line of sight. For the dimensions inferred from Fig. 1, $\alpha = -1.5$ and upper and lower synchrotron-radiation cutoff frequencies of 10 and 100,000 MHz, the equipartition magnetic field strength of the bridge can be expressed as

$$B_{\text{eq}} = 0.21 \left(\frac{S}{0.75 \text{ Jy}} \right)^{2/7} (1+k)^{2/7} \phi^{-2/7} \left(\frac{d}{1 \text{ Mpc}} \right)^{-2/7} \mu\text{G} \quad (1)$$

If the radiating volume increases by the (unknown) depth d , the associated equipartition magnetic field is lower by only $d^{-2/7}$. The equipartition field strength is similarly only weakly sensitive to ϕ and k . We note that B_{eq} will increase above $0.25 \mu\text{G}$ if the filling factor is less than unity, and/or if the energy density of

intergalactic high-energy protons is significantly greater than that for the relativistic electrons.

Recent evidence from EXOSAT X-ray data¹⁶ and from a study of extended radio galaxies near Coma A (ref. 17) indicate that the ambient intergalactic thermal pressure is sufficient to confine the bridge emission that we have detected. The radiating lifetime of a confined, synchrotron-emitting gas at 326 MHz ($E \approx 10$ GeV) is $1-2 \times 10^8$ yr for magnetic field values in the range $0.2-0.6 \mu\text{G}$, which is determined by synchrotron and, to a greater degree, inverse Compton losses that are due to scattering off microwave-background photons. We note that the inverse Compton mechanism might also be contributing to X-ray emission, in which case the implied thermal gas pressure could be somewhat lower.

The origin of these large-scale magnetic fields is not yet known. The field is probably much older than the trapped electrons, which are probably produced by the galaxy systems. The magnetic field that we are detecting is probably the amplified 'fossil' of a primordial magnetic field which may have played an important role in forming the giant voids and superclusters. The primordial magnetic field has been substantially amplified in the protogalactic medium as the galaxies and clusters evolved from their early state.

We assume, in the absence of turbulent field amplification, that the magnetic field on a large scale is characterized by the so-called 'frozen-in' field approximation. A compression of an originally smoothed-out protogalactic medium would change the strength of the primordial magnetic field B_0 to B through $B/B_0 = (n/n_0)^\gamma$. Here n and n_0 are the particle number densities of the corresponding media, which are related to their linear dimensions through $n \propto l^{-3}$. The power-law index γ characterizes the 'frozen-in' field compression, which is typically known to vary between $\frac{1}{2}$ and $\frac{1}{3}$ in the interstellar medium¹⁸. If the intracluster magnetic field strengths result from the compression of a primordial magnetic field, the field strengths on cluster and galaxy scales, ($l \approx 10$ kpc and 1 Mpc respectively) should differ by a factor $\geq 10^2$. Taking the typical strength of a galactic magnetic field as 10^{-6} G, the intracluster magnetic field should, using this simple argument, be of the order of 10^{-8} G, and, in the region of the 'bridge', should be even smaller. This is clearly much less than the equipartition field strength inferred from the radio emission in the 'bridge' region, and supports the suggestion that substantial field amplification has occurred since the pre-galaxy epoch, that is, since the time when the voids were formed.

If the formation of the giant voids and superclusters was triggered by blast waves at an early stage of galaxy formation, such as in superconducting cosmic string theory¹⁹⁻²¹ or cosmic explosions^{22,23}, the amplification of any weak primordial fields

should involve turbulence. Furthermore²⁴⁻²⁶, if a turbulent intra-cluster magnetic field is generated in the wakes of galaxies, the magnetic energy is drawn from the kinetic energy of plasma motion. Mechanisms of this type are in any case much more efficient field amplifiers than straightforward field compression^{27,28}.

The large extent and the strength of the intergalactic magnetic fields revealed in Fig. 1 suggest that magnetic fields had a more important role than previously believed in the formation of galaxies and of larger hierarchical structures such as superclusters and voids. □

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- Chincarini, G. & Rood, H. J. *Astrophys. J.* **206**, 30-37 (1976).
- Tift, W. G. & Gregory, S. A. *Astrophys. J.* **205**, 696-708 (1976).
- Davis, M., Groth, E. J. & Peebles, P. J. E. *Astrophys. J.* **212**, L107-L111 (1977).
- Oort, J. H. A. *Rev. Astr. Astrophys.* **21**, 373-428 (1983).
- Zel'dovich, Ya. B., Einasto, J. & Shandarin, S. F. *Nature* **300**, 407-413 (1982).
- de Lapparent, V., Geller, M. J. & Huchra, J. P. *Astrophys. J.* **302**, L1-L5 (1986).
- Andernach, H., Ferretti, L. & Giovannini, G. *Astr. Astrophys.* **133**, 252-263 (1984).

- Willson, M. A. G. *Mon. Not. R. astr. Soc.* **151**, 1-44 (1970).
- Hanisch, R. J. *Astr. J.* **85**, 1565-1576 (1980).
- Jaffe, W. J., Perola, G. C. & Valentijn, E. A. *Astr. Astrophys.* **49**, 179-192 (1976).
- Giovannini, G., Ferretti, L. & Andernach, H. *Astr. Astrophys.* **150**, 302-306 (1985).
- Hanisch, R. J., Strom, R. G. & Jaffe, W. J. *Astr. Astrophys.* **153**, 9-12 (1985).
- Kim, K.-T., Kronberg, P. P., Dewdney, P. E. & Landecker, T. L. *Astrophys. J.* (in the press).
- Noordam, J. E. & de Bruyn, A. G. *Nature* **299**, 597-600 (1982).
- Haslam, C. G. T., Salter, C. J., Stoffel, H. & Wilson, W. E. *Astr. Astrophys. Suppl.* **47**, 1-143 (1982).
- Hughes, J. P., Gorenstein, P. & Fabricant, D. *Astrophys. J.* **329**, 82-96 (1988).
- Venturi, T., Ferretti, L. & Giovannini, G. *Astr. Astrophys.* **213**, 49-60 (1989).
- Troland, T. H. & Heiles, C. *Astrophys. J.* **301**, 339-345 (1986).
- Witten, E. *Nucl. Phys.* **B249**, 557-592 (1985).
- Vilenkin, A. *Phys. Rep.* **121**, 263-315 (1985).
- Ostriker, J. P., Thompson, C. & Witten, E. *Phys. Lett.* **B180**, 231-239 (1986).
- Ostriker, J. P. & Cowie, L. L. *Astrophys. J.* **243**, L127-L131 (1981).
- Ikeuchi, S. *Publ. astr. Soc. Japan* **33**, 211-222 (1981).
- Livio, M., Regev, O. & Shaviv, G. *Astr. Astrophys.* **70**, L7-L9 (1978).
- Jaffe, W. J. **241**, 925-927 (1980).
- Roland, J. *Astr. Astrophys.* **93**, 407-410 (1981).
- Vainshtein, S. I. & Zel'dovich, Ya. B. *Soviet Phys. Usp.* **15**, 159-172 (1972).
- De Young, D. S. *Astrophys. J.* **241**, 81-97 (1980).

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Low magnetic flux noise observed in laser-deposited *in situ* films of YB₂Cu₃O₇ and implications for high-*T_c* SQUIDS

M. J. Ferrari*, Mark Johnson*, Frederick C. Wellstood*, John Clarke*§, A. Inam†, X. D. Wu†, L. Nazar‡ & T. Venkatesan‡

* Department of Physics, University of California, Berkeley, California 94720, USA, and Center for Advanced Materials, Materials and Chemical Sciences Division, Lawrence Berkeley Laboratory, 1 Cyclotron Road, Berkeley, California 94720, USA

† Department of Physics, Rutgers University, Piscataway, New Jersey 08854, USA

‡ Bellcore, Red Bank, New Jersey 07701, USA

THE first device made from a thin film of a high-transition-temperature (high-*T_c*) superconductor was a d.c. SQUID (superconducting quantum interference device)¹. Many applications of SQUIDS demand high sensitivity at low frequencies f (≤ 1 Hz), and thus require films with low intrinsic $1/f$ magnetic flux noise^{2,3}. Although the level of $1/f$ noise in Ti₂Ba₂Ca₂Cu₃O_x SQUIDS⁴ is significantly lower than that in earlier YBa₂Cu₃O₇ (YBCO) SQUIDS¹, it remains higher than that in low-*T_c* devices. In post-annealed films of YBCO, the magnitude of the $1/f$ noise has been shown⁵ to decrease dramatically as the quality of the films is improved. Here we report measurements of flux noise in a film of YBCO grown *in situ* by pulsed laser deposition (but not patterned into a SQUID). The $1/f$ noise level was two orders of magnitude lower than in our best post-annealed film. Provided that it proves possible to fabricate suitable low-noise Josephson junctions, a d.c. SQUID made from a film of this quality and operated at liquid-nitrogen temperature (77 K) should approach the low-frequency performance currently achieved in commercially available low-*T_c* SQUIDS.

The YBCO film was deposited on a (100) LaAlO₃ substrate using 30-ns pulses of 248-nm ultraviolet radiation from a KrF excimer laser^{6,7} with an energy density of about 2 J cm⁻² incident on a rotating stoichiometric target. The substrate was maintained at 650-750 °C to ensure *in situ* crystallization of the film in the ambient oxygen pressure of 0.2 torr. Figure 1 shows Rutherford backscattering spectra obtained from the as-grown film. These

results indicate that the film has a thickness of 300 nm, the correct stoichiometry, nearly perfect *c*-axis alignment (as verified by X-ray diffraction), and a density of macroscopic defects comparable with bulk single crystals⁸. The critical current density in zero field, defined as the value at an electric field of 1 μ V cm⁻¹, was 5×10^6 A cm⁻² at 77 K and 2×10^7 A cm⁻² at 4.2 K; these high values may be the result of strong flux pinning by microstructural defects⁹. Electron-microscope images revealed that the surface was very smooth (surface roughness < 5 nm) except for the presence of particles of ~ 1 - μ m diameter on the surface with a density of about one per 100 μ m².

We measured the flux noise with a Nb-PbIn thin-film d.c. SQUID¹⁰ attached to a sapphire plate maintained at 4.2 K inside a vacuum can⁵. The YBCO film was mounted on a thermally isolated hot stage parallel to and approximately 100 μ m away from the SQUID. The temperature of the stage could be

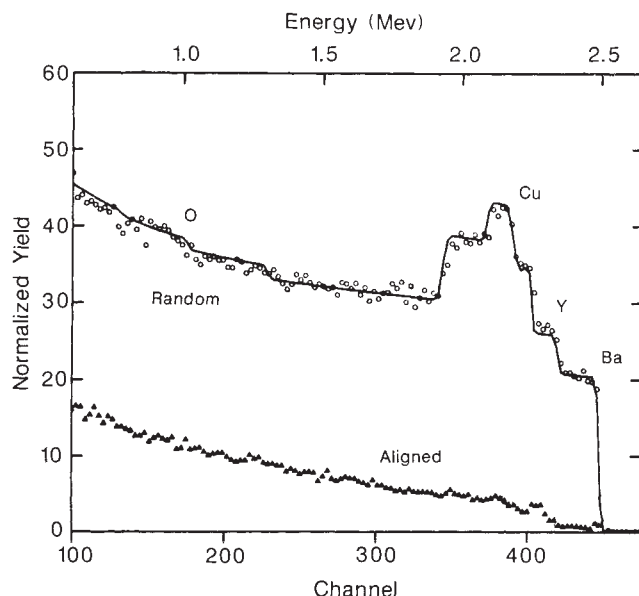


FIG. 1 Random (upper curve) and channelling (lower curve) Rutherford backscattering spectra obtained with 2.8-MeV He²⁺ ions from the as-grown film of YBCO. The fit to the random backscattering spectrum (solid line) corresponds to a film 300 nm thick and with correct stoichiometry. When the ion beam is aligned with the substrate (100) direction, the number of backscattered ions drops substantially. The ratio of backscattered yield from random and aligned spectra measured just below the Ba surface peak, about 3%, is similar to that obtained for single crystals.

§ To whom correspondence should be addressed.

regulated at any value between 4.2 and 140 K. Magnetic flux noise in the YBCO film was inductively coupled to the SQUID with an efficiency of $\sim 70\%$. We determined the critical temperature of the film by measuring the applied magnetic field B_0 required to produce one flux quantum (ϕ_0) in the SQUID; the diamagnetic screening of the superconducting film increased the value of B_0 over that in the normal state. We find that $T_c \approx 89.5$ K. A mu-metal shield around the cryostat reduced the ambient magnetic field below 10^{-6} tesla.

Figure 2 shows the spectral density of the flux noise, $S_\phi(f)$, at five temperatures. At 4.2 K, the noise is nearly white and is dominated by noise in the SQUID. At 77.4 K the spectral density of the low-frequency noise in the film has increased by a factor of about four compared with the 4.2-K value. The spectra at the three higher temperatures are $1/f$ -like, with the noise increasing rapidly with temperature. We believe that the noise arises from the thermally activated hopping of flux quanta (or possibly bundles of quanta) among pinning sites in the film; this motion induces flux noise in the measuring SQUID. If we assume that we can characterize each hopping process by a single, temperature-dependent hopping time τ , the corresponding spectral density of the noise will be a lorentzian of the form $\tau/[1+(2\pi f\tau)^2]$. Provided that these processes are statistically independent and have a suitably broad distribution of characteristic times, their superposition yields a spectral density that scales as $1/f^\alpha$, where $\alpha \approx 1$ (ref. 11). The increase in the noise magnitude (Fig. 2) with temperature is qualitatively consistent with a thermal-activation model. We note also that the slope α varies with temperature, reflecting the fact that different hopping processes contribute to the noise as the temperature is changed.

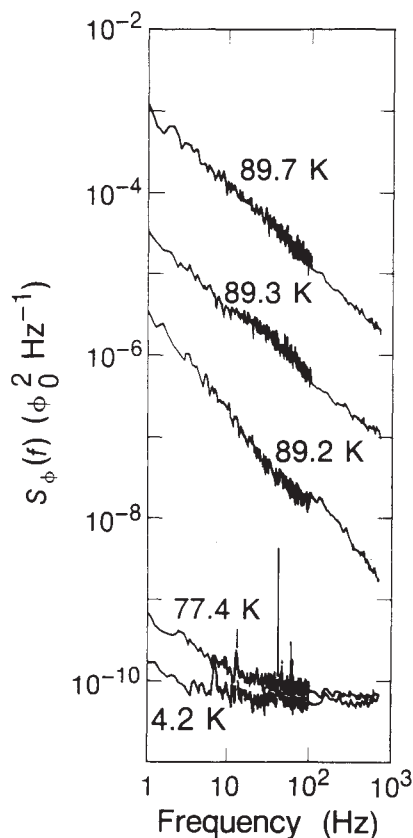


FIG. 2 Spectral density $S_\phi(f)$ of flux noise in a YBCO film at five temperatures. The spectral density detected by the d.c. SQUID has been multiplied by a factor of two to account for coupling losses between the film and the SQUID. At 4.2 K, the observed noise can be attributed to the SQUID measuring system; the SQUID contribution may be significant at temperatures up to 87 K. Spikes are from 60-Hz pick-up and microphonics.

In Fig. 3, we plot S_ϕ (1 Hz) against T . The noise rises slowly as temperature increases from 4.2 K to 87 K, and then rises steeply towards a very sharp peak at T_c . At 75 K, however, there is a small peak at 75 K that we have observed previously in another YBCO film⁵, which we tentatively associate with a pinning mechanism, so far unidentified, with a particular value of pinning energy. To illustrate the behaviour near T_c , in Fig. 4 we plot B_0 and S_ϕ (1 Hz) on an expanded temperature scale. The noise peaks at the temperature where diamagnetic screening vanishes. The open circles indicate temperatures at which the noise was dominated by a single process in which a flux quantum switched back and forth between two states. This process was clearly visible in the time domain, and produced a lorentzian power spectrum. As the temperature was raised, the frequency of the switching event increased and its contribution to the noise at 1 Hz decreased in a manner consistent with thermal activation.

The inset of Fig. 3 shows the variation of $fS_\phi(f)$ with B , the perpendicular magnetic field in which the film was cooled. Although not apparent in this figure, the values of $fS_\phi(f)$ for zero applied field lie above the linear extrapolation to $B=0$. We expect the number of flux quanta in the film to be proportional to B , so that if they moved coherently the noise power should scale as B^2 . On the other hand, if the vortices move incoherently, the noise power should be proportional simply to the number of vortices, that is, to B . The fact that $fS_\phi(f)$ is roughly linear in B for $B \leq 1.5$ mT implies that the interaction among the vortices producing the noise is negligible. Needless to say, there may be many vortices that actually interact strongly and as a result do not move or contribute to the noise. We note that at the highest applied field, 1.5 mT, the average vortex spacing of roughly $1 \mu\text{m}$ is greater than the vortex diameter of about one penetration depth¹² (~ 0.2 and $0.35 \mu\text{m}$ at 74 and 85 K, respectively), although in the presence of pinning the vortices need not be uniformly distributed.

Our upper bound on the spectral density of the flux noise of this *in situ* film between 70 and 80 K (Fig. 3), about $8 \times 10^{-10} \phi_0^2 \text{ Hz}^{-1}$ at 1 Hz, is two orders of magnitude lower than that measured previously in our best post-annealed film⁵. There is no reason to believe that this value represents a minimum: further improvements in the quality of films may well yield even lower flux noise. This noise is low enough, however, to have significant implications for SQUID magnetometers. If we express the spectral density as a noise energy $\varepsilon(1 \text{ Hz}) = S_\phi(1 \text{ Hz})/2L$ for a SQUID with inductance $L = 100$ pH (for

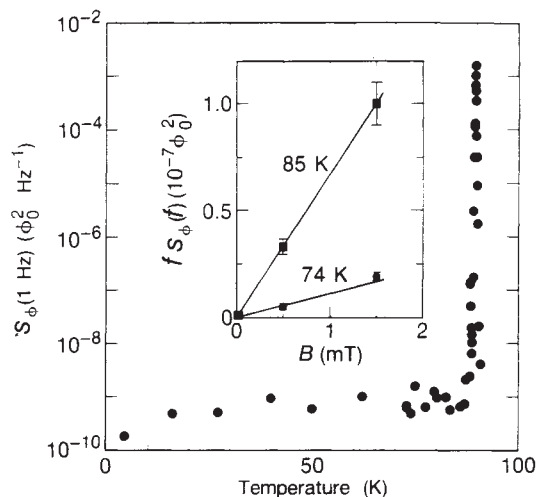


FIG. 3 Flux noise $S_\phi(1 \text{ Hz})$ against temperature for a YBCO film in zero applied field. Values below $8 \times 10^{-10} \phi_0^2 \text{ Hz}^{-1}$ include significant contributions from the measuring system, and therefore represent upper limits on noise from the film. Inset shows $fS_\phi(f)$ ($3 \text{ Hz} \leq f \leq 30 \text{ Hz}$) at two temperatures as a function of the perpendicular magnetic field B in which the film was cooled. Lines are guides to the eye.

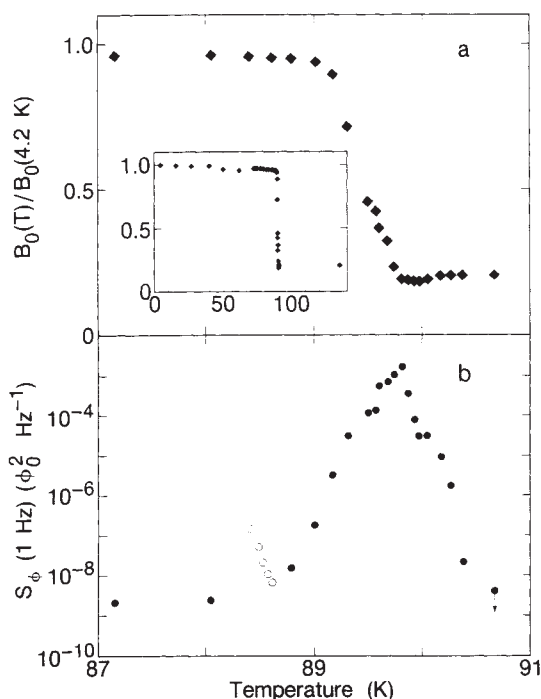


FIG. 4 *a*, Normalized applied magnetic field B_0 required to generate one flux quantum in the d.c. SQUID against temperature near T_c . The drop in B_0 reflects vanishing diamagnetic screening as the YBCO film becomes normal. Inset shows B_0 against T over the range 4.2–140 K. *b*, $S_\phi(1 \text{ Hz})$ against T near T_c . Solid symbols: $1/f$ spectra; open symbols: Lorentzian spectra from bistable switching; downward arrow: upper limit on noise.

example), we find $\varepsilon(1 \text{ Hz}) \approx 1.7 \times 10^{-29} \text{ J Hz}^{-1}$. Compared with the noise energies of commercially available devices operated at 4.2 K, this value is only a factor of six greater than that of d.c. SQUIDS and a factor of two less than r.f. SQUIDS. At 10 Hz, the noise energy in our sample (including contributions from the measuring SQUID) is about $3 \times 10^{-30} \text{ J Hz}^{-1}$, roughly 20 times less than the lowest value yet reported in a high- T_c SQUID of comparable inductance⁴. Of course, low flux noise is not in itself sufficient for low noise in a SQUID; one also has to make two junctions with sufficiently low white noise and $1/f$ noise. We note that one cannot make grain-boundary junctions in these high-quality films, and new technologies such as layered junctions (C. T. Rogers *et al.*, unpublished work) or edge junctions (R. H. Koch, Int. Conf. on Materials and Mechanisms of Superconductivity, Stanford, 1989) will be required. But it is most encouraging that a white-noise energy of $\sim 2 \times 10^{-30} \text{ J Hz}^{-1}$ has already been reported in the quietest high- T_c d.c. SQUID produced so far⁴. Furthermore, if $1/f$ noise in the junctions arises from critical-current fluctuations, it can be reduced substantially by a double-modulation scheme^{13,14}. Finally, our results also imply that flux transformers made from high-quality films should not induce significant levels of low-frequency noise into the SQUID. However, operation of either the SQUID or the flux transformer in modest ambient magnetic fields is likely to increase the level of low-frequency noise. □

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- Koch, R. H., Umbach, C. P., Clark, G. J., Chaudhari, P. & Laibowitz, R. B. *Appl. Phys. Lett.* **51**, 200–202 (1987).
- Clarke, J. *Nature* **333**, 1–7 (1988).
- Clarke, J. & Koch, R. H. *Science* **242**, 217–223 (1988).
- Koch, R. H., Gallagher, W. J., Bumble, B. & Lee, W. Y. *Appl. Phys. Lett.* **54**, 951–953 (1989).
- Ferrari, M. J. *et al. Appl. Phys. Lett.* **53**, 695–697 (1988).
- Inam, A. *et al. Appl. Phys. Lett.* **53**, 908–910 (1988).
- Venkatesan, T. *et al. Appl. Phys. Lett.* **54**, 581–583 (1989).
- Stoffel, N. G., Morris, P. A., Bonner, W. A. & Wilkens, B. J. *Phys. Rev. B* **37**, 2297–2300 (1988).
- Gregory, S. *et al. Phys. Rev. Lett.* **62**, 1548–1551 (1984).
- Wellstood, F. C., Heiden, C. & Clarke, J. *Rev. Sci. Instrum.* **55**, 952–957 (1984).
- Dutta, P., Dimon, P. & Horn, P. M. *Phys. Rev. Lett.* **43**, 646–649 (1979).
- Fiory, A. T., Hebard, A. F., Mankiewich, P. M. & Howard, R. E. *Phys. Rev. Lett.* **61**, 1419–1422 (1988).
- Koch, R. H. *et al. J. low-temp. Phys.* **51**, 207–224 (1983).
- Foglietti, V. *et al. Appl. Phys. Lett.* **49**, 1393–1395 (1986).

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Are classical weak-link models adequate to explain the current-voltage characteristics in bulk $\text{YBa}_2\text{Cu}_3\text{O}_7$?

S. S. Bungre*, R. Meisels†, Z. X. Shen* & A. D. Caplin*

* Centre for High Temperature Superconductivity, Blackett Laboratory, Imperial College, London SW7 2BZ, UK

† Institut für Festkörperphysik der Universität Wien, Kopernikusgasse 15, A1460 Wien, Austria

THERE is now a consensus that in bulk high-temperature superconductors, it is the grain boundaries that limit the attainable critical current. Their behaviour resembles that of low-temperature superconductors containing 'weak links'^{1,2} (regions of suppressed superconducting order). Here we report detailed measurements of the current-voltage characteristics of $\text{YBa}_2\text{Cu}_3\text{O}_7$, and find that the critical currents are too low, by two or three orders of magnitude, to be straightforwardly accommodated by classical weak-link models. This discrepancy is sufficiently fundamental to suggest that weak-link superconductivity in high-temperature superconductors has yet to be described adequately.

When the current through a weak link in a low-transition-temperature (low- T_c) superconductor greatly exceeds its critical current i_c , the slope dV/di of the current-voltage (I - V) characteristic is just the normal-state resistance r_n of the link. With some exceptions (to be discussed below), the zero-temperature critical current $i_c(0)$ is related to r_n by

$$r_n i_c(0) \sim \Delta(0)/e \quad (1)$$

where $\Delta(0)$ is the zero-temperature superconducting energy gap, and e is the electronic charge. The behaviour of arrays of weak links depends greatly on their dimensionality; two-dimensional arrays fabricated from low- T_c materials have been studied extensively³, but their critical currents are limited by the onset of the vortex-unbinding (Kosterlitz-Thouless⁴) transition, rather than by equation (1). Very little work has been done on three-dimensional arrays of these materials, for which vortex unbinding should not occur⁵; granular niobium composites⁶, however, show behaviour that is consistent with equation (1) within an order of magnitude or so. In bulk sintered high- T_c superconductors, the granular weak-link network is certainly three-dimensional. We have examined the I - V characteristics of several bulk samples of high-quality $\text{YBa}_2\text{Cu}_3\text{O}_7$ at currents well above critical, using a sensitive pulsed-current system that eliminates the effects of joule heating⁷.

Figure 1*a* shows typical I - V curves in zero applied field (the ambient magnetic field is about 50 μT); their linearity at high currents is immediately apparent. As the temperature is lowered, the critical current increases and at about 50 K it reaches the maximum available current of 10 A. Small magnetic fields depress the critical current sufficiently to allow the characteristics to be measured to lower temperatures (Fig. 1*b*) while altering the I - V slopes only slightly. In this range of field there is little difference between the effects of fields transverse and parallel to the current, and no irreversibility with respect to field cycling.

The data are summarized in Fig. 2, where the I - V -slope resistivities ρ' , which we will relate to the weak-link resistance

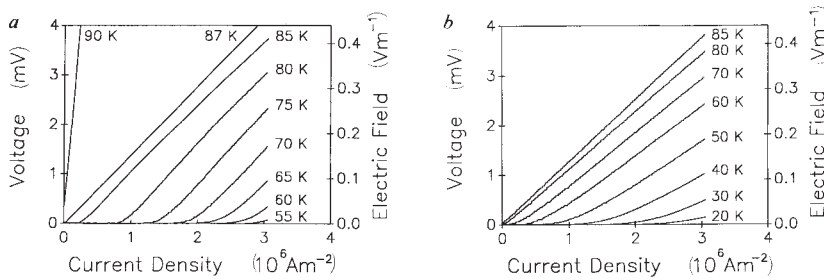


FIG. 1 I - V characteristics of sample MS95/4 in *a*, the ambient field of $50 \mu\text{T}$, and *b*, a transverse applied field of 2.3 mT . The linear regions reflect dissipation in the inter-granular weak links.

r_n , are plotted and compared with the resistive transition measured at low current ($\sim 1 \text{ mA}$). On cooling through the superconducting transition, ρ' first follows the low-current resistivity ρ extremely closely, but at the lower end of the transition it levels off to a small fraction of $\rho(100)$, the normal-state resistivity at 100 K .

Before discussing the significance of the magnitude of ρ' , a number of other observations deserve comment. $\text{YBa}_2\text{Cu}_3\text{O}_7$ samples of lesser quality (data not shown), which consequently have much higher normal-state resistivities, behave in a qualitatively similar way except that at low temperatures ρ' levels off to a much larger fraction of $\rho(100)$, suggesting that the extra normal-state resistivity arises from weak links of greater resistance. Preliminary results on a $\text{Bi}_2\text{Sr}_2\text{Ca}_2\text{Cu}_3\text{O}_x$ sample resemble those of poor-quality $\text{YBa}_2\text{Cu}_3\text{O}_7$. Below T_c , ρ' depends only weakly on temperature and field (Fig. 2*b*), which will be discussed in detail elsewhere. Qualitatively, the maximum in ρ' at $\sim 73 \text{ K}$ that occurs in zero applied field can be understood in terms of the current distribution over the sample cross-section: immediately below T_c , the penetration depth λ_{net} of the granular network⁸ is large compared with the sample diameter, so that the current is distributed uniformly and ρ' represents simply the macroscopic series-parallel combination of weak-link resistances. As the temperature is lowered, λ_{net} diminishes, the current is carried non-uniformly and the macroscopic resistance increases. A small field not only depresses the critical current density J_c but also increases λ_{net} and so restores uniformity to the current distribution, thereby decreasing ρ' in this temperature range. The decrease of ρ' in zero applied field at lower temperatures reflects the intrinsic temperature variation of the weak-link resistance r_n , and indicates that the weak links are metallic rather than semiconducting.

Returning now to the central question of the magnitude of ρ' , we model the material as a network of identical weak links, each having normal-state resistance r_n and critical current i_c , forming a cubic array of cell size d (a typical grain size); we make no other assumptions about the geometry. Clearly, $r_n = \rho'/d$ and $J_c = i_c/d^2$. The grain size is $\sim 10 \mu\text{m}$, so that, just below T_c , $r_n \approx 20 \mu\Omega$. We estimate⁶ $J_c(0) \approx 10^7 \text{ A m}^{-2}$, so that $i_c(0) \approx 1 \text{ mA}$. Consequently, the product $r_n i_c(0)$ is not more than $20 \mu\text{V}$; a $\text{YBa}_2\text{Cu}_3\text{O}_7$ sample of somewhat smaller grain size (data not shown) had larger ρ' and J_c , again leading to estimates of $r_n i_c(0)$ of about $20 \mu\text{V}$. Self-field effects may have led us to somewhat underestimate J_c ; on the other hand, we have used the value of r_n obtained just below T_c , and it decreases with temperature. We can be confident that the low-temperature limit of $r_n i_c(0)$ is

not greater than $100 \mu\text{V}$. By contrast, the T_c of 92 K suggests that the energy gap in $\text{YBa}_2\text{Cu}_3\text{O}_7$ is $\sim 20 \text{ meV}$, so that there is a discrepancy of two to three orders of magnitude with equation (1). In experiments⁹ on bi-crystals that share a common c -axis (and thus for which current flows in the favourable a - b plane), the grain boundary weak links were found to have $r_n i_c(0)$ values in the range 0.3 to 3 mV .

There are two important situations in which $r_n i_c(0)$ for a microscopically uniform weak link can be much less than $\Delta(0)/e$, namely for a superconductor-normal-superconductor (proximity-effect) junction with a normal region many coherence lengths thick, and for a wide junction. In the former case, it is well-known that J_c depends exponentially on temperature^{4,10}, in clear contrast to the much weaker dependence that is observed in the new materials^{6,8}. For a junction whose width w is large compared with the Josephson penetration depth λ_J , the average critical current density j_{av} is $\sim (2\lambda_J/w)j_c$, where j_c is the critical current density of a narrow junction. λ_J is given by¹¹ $\lambda_J^2 = \Phi_0/(4\pi\mu_0 j_c)$, where λ is the usual penetration depth. j_c can be eliminated, and the product $(\lambda_J w)$ estimated from the measured (average) critical current density J_c and λ ($\sim 0.3 \mu\text{m}$). We find $(\lambda_J w) \approx 100 \mu\text{m}^2$. Because w cannot exceed the grain size ($\sim 10 \mu\text{m}$), λ_J cannot be less than w , and so we can discard the possibility that the junctions are wide. We may note also that if an additional intergranular flux flow term is contributing to our inferred value of r_n , the discrepancy with equation (1) becomes even worse.

Thus, either the theory of classical weak links is inapplicable to grain boundaries in high- T_c superconductors (which, given the extreme shortness of the coherence length ξ , needs to be considered), or the supercurrent transport across grain boundaries is microscopically inhomogeneous, as has been suggested by Larbalestier and co-workers on the basis of microstructural studies and the temperature dependence of the critical current^{12,13}. For example, it might be that, at any one grain boundary, supercurrent flows across only a much smaller fraction of the interface area than does normal current; the resistance of the latter would shunt that of the former and thereby reduce the observed values of r_n and $r_n i_c(0)$. Alternatively, the disproportionation between super- and normal currents could occur between different orientations of grain boundaries. In either case, equation (1) would no longer apply. The breakdown of equation (1) in the high- T_c materials is of considerable fundamental significance. In low- T_c superconductors, the relationship is so general because it reflects the equality of the matrix elements that control the transmission of pairs (in the superconducting state) and of electrons (in the normal state) across the weak

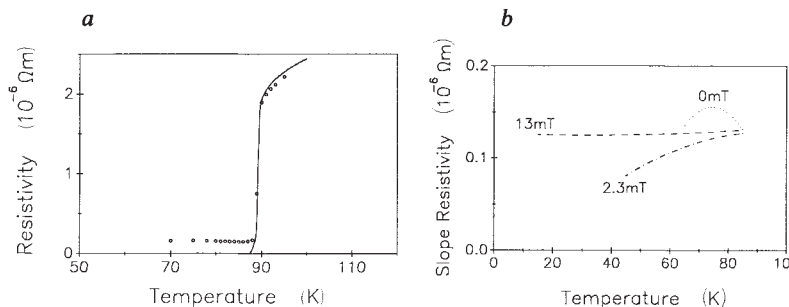


FIG. 2 *a*, Circles, high-current slope resistivities ρ' derived from I - V characteristics, as in Fig. 1; solid line, low-current resistivity ρ . *b*, ρ' on an expanded scale, showing the weak dependence on temperature below T_c and the effect of small applied fields.

link; it depends upon neither the superconducting mechanism nor the details of the weak link. The low critical currents across grain boundaries in high- T_c superconductors suggest that the situation is more complicated than that in the low- T_c materials. □

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1. Likharev, K. K. *Rev. mod. Phys.* **51**, 101–159 (1979).
2. Nicolsky, R. *Cryogenics* **29**, 388–391 (1989).
3. Lobb, C. J., Abraham, D. W. & Tinkham, M. *Phys. Rev. B* **27**, 150–157 (1983).
4. Kosterlitz, J. M. & Thouless, D. J. *J. Phys. C* **6**, 1181–1203 (1973).
5. England, P., Goldie, F. & Caplin, A. D. *J. Phys. F* **17**, 447–458 (1987).
6. Raboultou, A., Peyral, P. & Rosenblatt, J. in *Inhomogeneous Superconductors* (AIP Conf. Proc. No. 58, ed. Gubser, D. U. et al., 1980).
7. Meisels, R., Bunge, S. & Caplin, A. D. *J. less-common Metals* **151**, 83–88 (1989).
8. Male, S. E. et al. *Supercond. Sci. Technol.* **2**, 9–16 (1989).
9. Mannhart, J. et al. *Phys. Rev. Lett.* **61**, 2476–2479 (1988).
10. Hsiang, T. Y. & Finnemore, D. K. *Phys. Rev. B* **22**, 154–163 (1980).
11. Tinkham, M. *Introduction to Superconductivity* (McGraw-Hill, New York, 1975).
12. Larbalestier, D. C. et al. in *Proc. Tokai Univ. Symp. Nov. 1987* (ed. S. Nakajima) (World Scientific, Singapore, 1989); *High- T_c Superconductivity* **18**, 128 (1989).
13. Babcock, S. E. & Larbalestier, D. C. *Appl. Phys. Lett.* **55**, 393–395 (1989).

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Separation of nitrogen and oxygen isotopes by liquid chromatography

Nobuo Tanaka, Ken Hosoya, Kazuhiro Nomura, Tadanori Yoshimura, Takehiro Ohki, Ryohei Yamaoka*, Kazuhiro Kimata & Mikio Araki

Department of Polymer Science and Engineering, and * Department of Applied Biology, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606, Japan

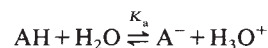
ISOTOPIC enrichment of organic compounds in the stable minor isotopes ^{15}N , ^{17}O and ^{18}O , which are often used as tracers, is commonly achieved by syntheses that involve isotopically enriched simple precursors such as NH_3 , H_2O and CO_2 . But such labelled compounds are not always commercially available or readily synthesized. Here we report the direct enrichment of nitrogen and oxygen isotopes in dissociable compounds such as amines, phenols and carboxylic acids, using a high-performance liquid-chromatography technique. The separation method relies on the isotope effects on the dissociation equilibria of weak acids and bases that occur in protonation or deprotonation of the relevant isotopes. Enrichment of ^{15}N to 5–10% isotopic purity in N,N -dimethylaniline was achieved from the natural abundance of 0.37% in less than 14 hours; subsequent runs brought the isotopic purity to 90% or more. Enrichment of ^{17}O and ^{18}O to similar high purities in p -nitrophenol was also obtained. Thus, where suitable dissociation equilibria exist, direct isotopic enrichment is possible using standard techniques without the need to devise complex synthetic pathways.

In addition to the conventional isotope separation methods including chemical exchange, distillation and ion-exchange chromatography, laser-based methods have been intensively studied recently. With the exception of ion-exchange chromatography^{1–6}, these methods require special equipment for the isotopic enrichment of simple working substances, and the desired labelled compounds must be synthesized after enrichment. The advantages of the ionization-control method over the conventional ion-exchange chromatography^{1–6} include the availability of high-performance columns and the applicability for a wide range of compounds.

In reversed-phase liquid chromatography of ionizable compounds, the neutral form of the compound is retained much longer by the hydrophobic stationary phase than is the ionized

form⁷, which is much more hydrophilic. Accordingly an acid is retained much longer at lower pH, whereas an amine shows the opposite tendency. Figure 1 shows that the apparent pK_a values are 4.6 for N,N -dimethylaniline (DMA) and 7.1 for p -nitrophenol (PNP).

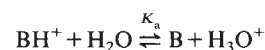
For the dissociation equilibria of an acid AH,



the chromatographic retention or capacity factor k' is⁷

$$k' = \frac{k'_{\text{AH}}[\text{AH}]}{[\text{AH}] + [\text{A}^-]} + \frac{k'_{\text{A}^-}[\text{A}^-]}{[\text{AH}] + [\text{A}^-]} \\ = \frac{k'_{\text{AH}}}{1 + K_a/[\text{H}_3\text{O}^+]} + \frac{k'_{\text{A}^-}K_a/[\text{H}_3\text{O}^+]}{1 + K_a/[\text{H}_3\text{O}^+]} \quad (1)$$

where k'_{AH} and k'_{A^-} are the retentions of the neutral and dissociated forms of the acid. Similarly, for the dissociation equilibrium of an amine B,



the chromatographic retention is

$$k' = \frac{k'_{\text{BH}^+}[\text{BH}^+]}{[\text{BH}^+] + [\text{B}]} + \frac{k'_{\text{B}}[\text{B}]}{[\text{BH}^+] + [\text{B}]} \\ = \frac{k'_{\text{BH}^+}}{1 + K_a/[\text{H}_3\text{O}^+]} + \frac{k'_{\text{B}}K_a/[\text{H}_3\text{O}^+]}{1 + K_a/[\text{H}_3\text{O}^+]} \quad (2)$$

where k'_{B} and k'_{BH^+} are the retentions of the neutral and protonated forms of the amine. The extent of ionization determines the retention of the ionizable species at particular pH in the ionization-control mode of reversed-phase liquid chromatography.

As the dissociation of a proton bound to a heavier isotope is slightly less favourable than that of a proton bound to a lighter isotope, the dissociation constant K_a is smaller for species with

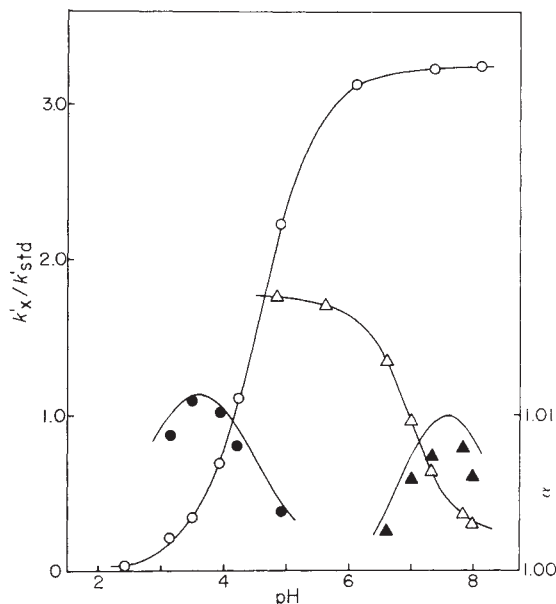


FIG. 1 pH dependence of k' values of DMA- ^{14}N (○) and PNP- ^{16}OH (△), and the pH dependence of isotopic separation factors α between DMA- ^{14}N and DMA- ^{15}N (●) and between PNP- ^{16}OH and PNP- ^{18}OH (▲). The k' values of DMA and PNP were normalized by using the k' values of 3-phenylpropanol and benzyl alcohol, respectively¹³. Curves were drawn based on the results obtained with the individual isotopic compounds. Column: Cosmosil-5C₁₈, 5- μm particles (Nacalai-Tesque), 4.6-mm inner diameter, 30 cm (15 cm $\times$ 2). Mobile phase: buffer-acetonitrile (80%–20% (v/v)) with 0.01% triethylamine for DMA, and buffer-methanol (95%–5% (v/v)) for PNP. Temperature: 30 °C.

a heavier isotope^{8,9}. This isotope effect leads to the difference in the extent of ionization of the species depending on the isotope mass, which in turn leads to a difference in the chromatographic retention of the isotopic compounds at a pH near the pK_a where both two terms of the righthand side of equations 1 and 2 make significant contributions.

Figure 2a shows the separation of a standard mixture (1:1) of DMA-¹⁴N and DMA-¹⁵N at pH 3.8. We found the nitrogen isotope effects on the dissociation of protonated amines, $^{14}K_a/^{15}K_a$, to be 1.019 for aniline¹⁰ and 1.014 for DMA, in agreement with the reported values^{11,12}. A maximum chromatographic separation factor, $^{14}k'/^{15}k'$, of 1.011 was observed at pH 3.5–4.0 for the isotopic DMA.

To enrich ¹⁵N from the natural abundance of 0.37%, we collected ~5% of the total peak, including the fraction corresponding to DMA-¹⁵N, as shown in Fig. 2b. The rechromatography of the collected fraction in Fig. 2c shows that the isotopic purity of ¹⁵N, by the comparison with the standard chromatograms, is ~6%, which agrees well with the results of gas chromatography/mass spectrometry determination. We can obtain an isotopic purity as high as 90% by collecting an appropriate fraction of the chromatographic separation shown in Fig. 2c.

The separation of ¹⁶O and ¹⁸O requires a similar column efficiency as that required for the separation of ¹⁴N and ¹⁵N. We have found the oxygen isotope effects on the dissociation $^{16}K_a/^{18}K_a$ to be 1.020 for benzoic acid (BA)-¹⁶O₂ and BA-¹⁸O₂ (ref. 13), and 1.017 for PNP-¹⁶OH and PNP-¹⁸OH. This results in an isotope separation factor $^{18}k'/^{16}k'$ of 1.010 for PNP. About 800,000 theoretical plates are required to achieve complete resolution of PNP-¹⁶OH, PNP-¹⁷OH and PNP-¹⁸OH, which can be provided by a column system of 15–20 m. We show the separation of the three oxygen isotopes by recycle chromatography^{13,14} of PNP in Fig. 3. The results show that ¹⁷O, which is currently available at less than 60% isotopic purity in the form of H₂O, can be enriched to a very high isotopic purity (90% and above) by simple fractionation of the chromatographic

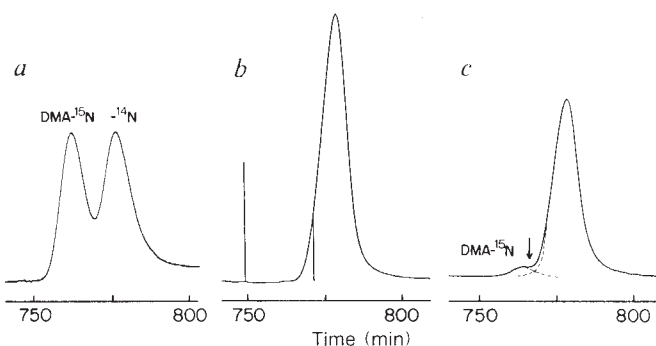


FIG. 2 Separation of DMA-¹⁴N and DMA-¹⁵N a, 1:1 mixture of DMA-¹⁴N and DMA-¹⁵N. b, Fractionation of DMA-¹⁵N from ordinary DMA. Every 40 min we injected 0.1 mg of ordinary DMA dissolved in 10 μ l of the mobile phase (The sample size and separation time depend on the column size and flow rate.) We collected the fraction between the two spikes, containing ~5 μ g of DMA, each time. We removed buffering species by chromatography of the combined collected fractions on a C₁₈ column using 60% methanol. Following the evaporation of methanol, we extracted the enriched material with ether. The gas chromatography/mass spectrometry analysis of the extracted DMA gave relative intensity, 100.00:80.75:11.11 for m/z values 120, 121 and 122 (M–1, M⁺, M+1), respectively, compared with 100.00:72.31:5.77 for ordinary DMA, indicating ~6.5% isotopic purity of ¹⁵N. c, Rechromatography of the separation products from b. Dashed lines indicate the simulated traces for DMA-¹⁵N and DMA-¹⁴N at a 6:94 ratio. We expect a 90% isotopic purity for DMA-¹⁵N, when the fraction is collected before the time marked by the arrow¹⁵. Column: Capcellpak C₁₈-SG, 5- μ m particles (Shiseido), 4.6-mm ID, 350 cm (10 $\times$ 15 cm + 8 $\times$ 25 cm). Mobile phase: 0.5M acetate buffer-acetonitrile (90%–10% (v/v)) with 0.01% triethylamine at pH 3.8. Flow rate: 0.37 ml min⁻¹. Temperature: 50 °C.

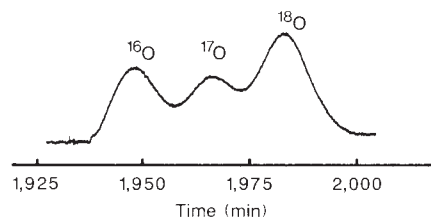


FIG. 3 Separation of PNP-¹⁶OH, PNP-¹⁷OH and PNP-¹⁸O. The solutes were recycled 12 times through a pair of 45-cm column systems by using an automated column-switching valve^{13,14}. Increases in the peak width and separation factor were observed because of the effect of the pressure gradient in the column¹⁶. We expect about 90% isotopic purity for PNP-¹⁷OH, if the fraction between the two minima were to be collected¹⁵. Column: Cosmosil-5C₁₈, 4.6-mm ID, 90 cm (6 $\times$ 15 cm). Mobile phase: 0.02M-phosphate buffer-methanol (95%–5% (v/v)) with 0.001M sodium octanoate at pH 7.6. Flow rate: 0.4 ml min⁻¹. Temperature: 40 °C. A mixture of ~0.5 μ g each of PNP-¹⁶OH, PNP-¹⁷OH and PNP-¹⁸OH was injected.

graphic effluent. The enrichment of ¹⁸O will be considerably easier than that of ¹⁷O because of its greater natural abundance.

In spite of the small isotope separation factors, the use of high-performance columns and optimized mobile phases has enabled us to achieve a fast separation of oxygen and nitrogen isotopes by reversed-phase liquid chromatography. By carrying out reversed-phase liquid chromatography at a pH value 0.5–1.0 pH units higher than the pK_a for weak acids or 0.5–1.0 pH units lower than the pK_a for weak bases, one can obtain enrichment of the ¹⁵N, ¹⁷O or ¹⁸O atoms involved in the dissociation equilibria. Current polymer-based packing materials allow operation of the chromatographic process in the range pH 2–12. □

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- Spedding, F. H., Powell, J. E. & Svec, H. J. *J. Am. chem. Soc.* **77**, 6125–6132 (1955).
- Gupta, A. R. & Sarpal, S. K. *J. phys. Chem.* **71**, 500–508 (1967).
- Kotaka, M., Shono, T. & Kakihana, H. *Chem. Lett.* 315–318 (1978).
- Trivelin, P. C. O., Matsui, E. & Salati, E. *Energia Nucleare e Agricoltura* **1**, 1–13 (1979).
- Hirschberg, K., Krumbiegel, P. & Faust, H. *Isotopenpraxis* **17**, 178–182 (1981).
- Ruiz Saenz de Miera, A. & Urgell, M. M. *An. Quim.* **A82**, 261–264 (1986).
- Karger, B. L., LePage, J. N. & Tanaka, N. in *High-Performance Liquid Chromatography* (ed. Horvath, C.) 113–206 (Academic, New York, 1980).
- Ellison, S. L. R. & Robinson, M. J. T. *JCS chem. Commun.* 745–746 (1983).
- Thornton, E. R. *J. Am. chem. Soc.* **84**, 2474–2475 (1962).
- Tanaka, N., Yamaguchi, A., Araki, M. & Kimata, K. *J. Am. chem. Soc.* **107**, 7781–7782 (1985).
- Hermes, J. D., Weiss, P. M. & Cleland, W. W. *Biochemistry* **24**, 2959–2967 (1985).
- Ando, T., Yamataka, H. & Wada, E. *Israel J. Chem.* **26**, 354–356 (1985).
- Tanaka, N., Araki, M. & Kimata, K. *J. Chromatogr.* **352**, 307–314 (1986).
- Henry, R. A., Byrne, S. H. & Hudson, D. R. *J. chromatogr. Sci.* **12**, 197–199 (1974).
- Snyder, L. R. & Kirkland, J. J. *Introduction to Modern Liquid Chromatography* 37–43 (New York, 1979).
- Tanaka, N. et al. *J. High Resolut. Chromatogr. Chromatogr. Commun.* **9**, 683–687 (1986).

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Biases in satellite-derived sea-surface-temperature data

Richard W. Reynolds*, Chris K. Folland† & David E. Parker†

* Climate Analysis Center, NMC/NWS/NOAA, Washington, DC 20233, USA
† UK Meteorological Office, Bracknell, Berkshire RG12 2SZ, UK

STRONG¹ indicates from satellite data that global sea surface temperature increased by 0.1 °C per year over the 6.5-year period between January 1982 and June 1988. Here we show that no significant trend can be seen in three analyses of global sea surface temperatures that are based on *in situ* data in this limited period, nor in an independent analysis of sea-surface-temperature (SST) and land-air-temperature data. Satellite data are blended and adjusted in one of the above SST analyses. An unadjusted satellite

SST analysis similar to that used by Strong shows the same, but probably spurious, trend.

Three of the SST analyses we use here were produced at the Climate Analysis Center (CAC) of the US National Meteorological Center, and comprise an *in situ*, a satellite and a blended analysis. The *in situ* SST analysis uses radio-transmitted data from ships and buoys. The satellite analysis uses the multichannel SST (MCSST) data reported by Strong, but with small differences in analytical procedure. The blended SST analysis combines satellite and *in situ* SST observations by using the *in situ* analysis to define 'ground truth' temperatures in regions of sufficient *in situ* data and the analysed satellite data to define the shape of the SST field where there are few or no *in situ* data². Where enough *in situ* observations are available, they provide all the information about SST; elsewhere the adjusted satellite field provides increasing information about SST patterns as *in situ* data become sparser.

We also use a quality-controlled *in situ* SST analysis (produced by the UK Meteorological Office (UKMO)^{3,4}, as well as a data set of air temperatures measured at night (UKMO). Both data sets use temperatures measured by ships and buoys. We excluded daytime air-temperature measurements because of the risk that variable biases could be introduced by overheating of the ship's thermometer housing by day⁵. In the regional time series, the grid-box data are weighted by the cosine of the latitude.

Figures 1a and 1b show monthly anomalies for the CAC SST analyses for January 1982–March 1989, averaged over two regions: Equator to 60° N (the same as Strong's Northern Hemisphere area), and 40° S to the Equator. The anomalies were calculated relative to a 1950–79 climatology. The southern limit of 40° S was selected for the second region because of the scarcity of *in situ* observations south of 40° S. The Northern Hemisphere satellite time series (light line) in Fig. 1a is similar to that shown by Strong (his Fig. 1b); the two series have the same linear trend of 0.16 °C yr⁻¹ between January 1982 and June 1988. The two satellite time series are not quite identical because CAC and Strong used different climatologies and averaged the MCSST data on different grids (2° and 2.5°, respectively). In addition, Strong averaged only grid values with data (that is, he did not interpolate data for grids where the satellite data were unavailable because of cloud cover) and did not use cosine weighting to reduce the weight of boxes at higher latitudes. He effectively assumed that all latitude zones over the ocean had the same longitudinal extent because he averaged zonal means of SST with equal weight for each zone (Strong, personal communication). The CAC technique interpolated data for missing grid points over the ocean and used all oceanic grid points (within the given latitudinal bounds) with cosine weighting. Although these analysis differences cause small monthly changes, major features, such as the above trends, were unaffected. (We note that the confidence limits quoted by Strong for his linear trends are too narrow as he did not account for the persistence of SST anomalies for periods longer than a month.)

Figure 1a also shows the CAC blended (thick line) and CAC *in situ* SST analyses (dots) for 0° to 60° N; the two data sets are nearly identical (correlation $r = 0.97$ for January 1982–March 1989). There is no significant trend in either set for January 1982–June 1988 or January 1982–March 1989. This agreement between *in situ* and blended SST anomalies demonstrates that the blending technique successfully adjusts the satellite data relative to the *in situ* analysis. The warmth of 1982–83 is mostly due to the 1982–83 El Niño. The uncorrected satellite data, however, indicate very low SSTs during this period (see ref. 6, comment on the same paper).

Figure 1b, for the Equator to 40° S, covers a smaller area than Strong's Fig. 1c (Equator to 60° S) but the trend of 0.12 °C yr⁻¹ in the CAC satellite data for January 1982–June 1988 is similar to Strong's value of 0.10 °C yr⁻¹ over his larger area. *In situ* data

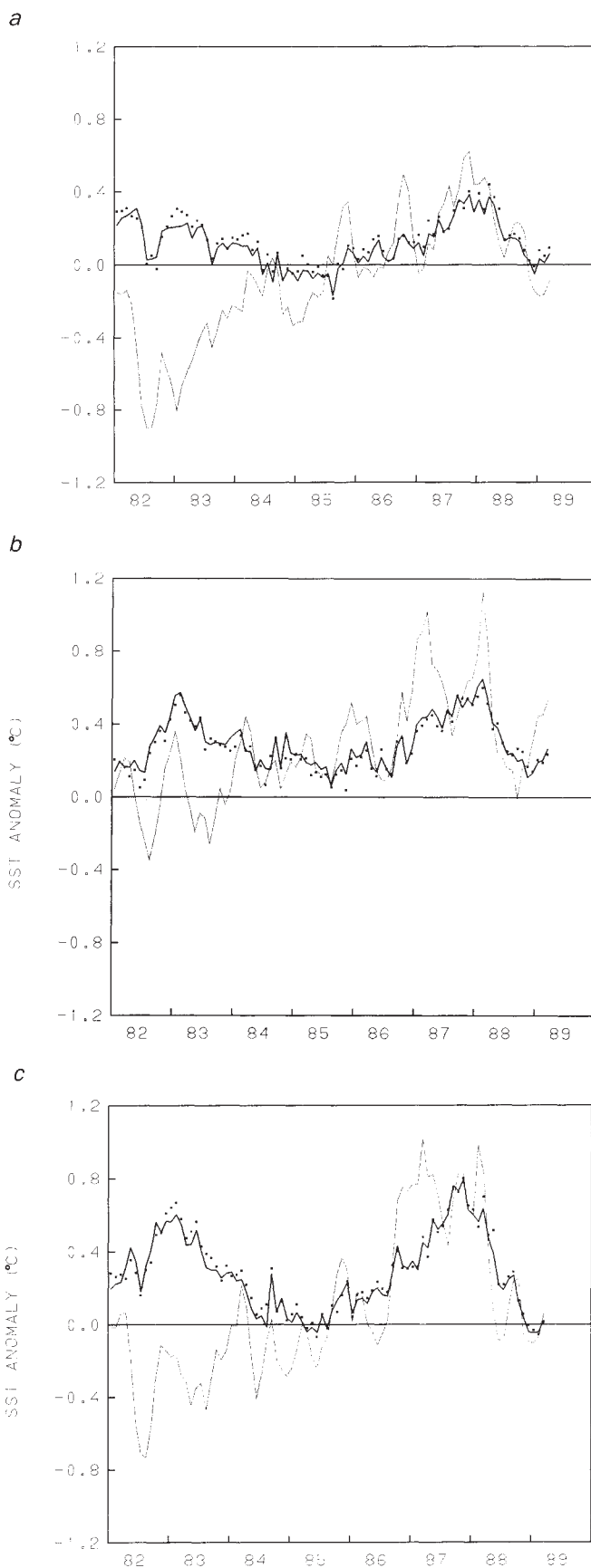


FIG. 1 a, Time series of CAC SST anomalies for the Northern Hemisphere between 0° and 60° N, January 1982–March 1989. Satellite anomalies are indicated by a light line; blended anomalies are indicated by a heavy line; *in situ* anomalies are dotted. b, As in a, except the region is 40° S to 0°. c, As in a, but for the region 20° S to 20° N.

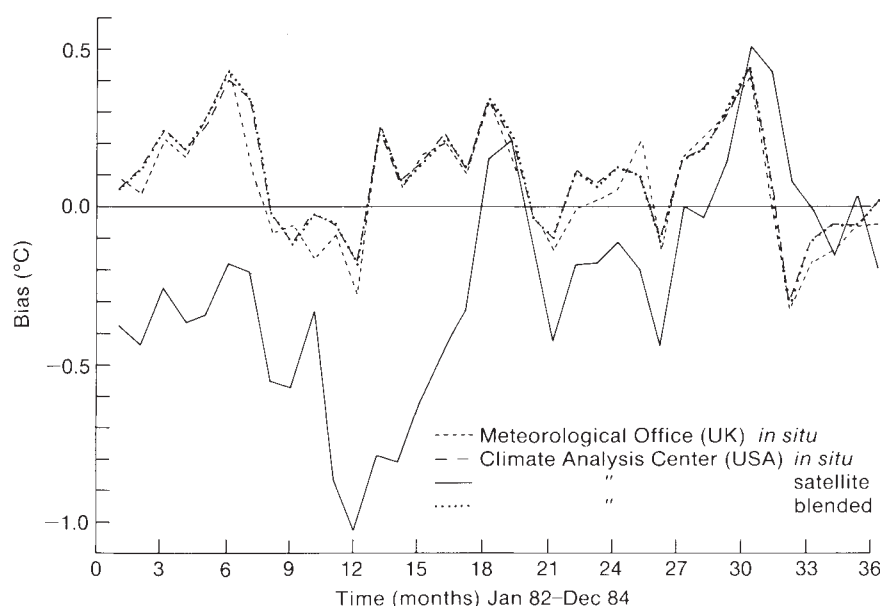


FIG. 2 Mean differences between UKMO *in situ*, CAC satellite, CAC *in situ* and blended analyses and quality-controlled expendable bathythermograph data for the region 0° to 60° N for January 1982–December 1984.

again show no significant trend. Agreement between the CAC blended and *in situ* analyses is again very good ($r=0.96$ for January 1982–March 1989).

Strong states that the warming trend in the satellite data is about twice that of a warming trend shown by *in situ* data sources. The Oort *et al.*⁷ data that he discusses in this connection extend only to 1985; for the period of overlap with Strong's record, Oort *et al.* use the CAC blended analysis (Figs. 1a and 1b), which, of course, includes adjusted satellite data. No significant global or hemispheric warming trend exists in the limited period of overlap with Strong's data (1982–1985). A linear trend fitted to the Oort *et al.* Northern Hemisphere data over this four-year period shows slight cooling.

To help clarify the relative accuracies of satellite and *in situ* SST data, we have compared them with expendable bathythermograph (XBT) data for January 1982–December 1984. Although the SSTs from XBTs are sparser than the conventional surface marine *in situ* observations used above (the ratio of number of XBTs to conventional SSTs is roughly 5%), they are useful because XBTs are not included in any of the analyses used here and are widely distributed spatially. Figure 2 shows time series of CAC satellite, CAC *in situ*, CAC blended and

UKMO *in situ* SST analyses for the Northern Hemisphere subtracted from quality-controlled near-surface SST data derived from XBTs. We call these differences 'biases'. Mean biases were 0.04 °C, 0.04 °C and 0.01 °C for CAC *in situ*, CAC blended and UKMO analyses respectively, and were not statistically significant. The mean bias for the satellite analysis was -0.30 °C and was highly significant, showing that the satellite-derived temperatures were almost certainly too low overall. Furthermore, the satellite bias varied from near -0.8 °C between mid-1982 and early 1983 to small values towards the end of 1984. Thus the satellite biases varied in such a way as to give rise to a pronounced spurious warming trend in 1982–84. In contrast, the XBT data support the lack of trend in the other SST analyses.

During the period of interest, two El Niño/Southern Oscillation events (1982–83 and 1986–87) caused strong warming of the tropical oceans, primarily in the central and east Pacific. Figure 1c shows time series of the monthly CAC satellite, *in situ* and blended analyses between 20° S and 20° N. These tropical warmings are shown in the *in situ* and blended analyses. As discussed by Robock⁶, El Chichón aerosols resulted in a negative bias in satellite SST retrievals from April 1982 to the end of 1983. Thus, the satellite analysis in Fig. 3 does not show the large-scale warming associated with the 1982–83 El Niño event. Despite this, closer analysis shows that the satellite retrievals did detect a locally strong but distorted El Niño warming. The warming observed in the satellite data (south of 5° N in the tropical east and central Pacific) was offset, however, not only by unrealistic negative biases north of this latitude in the Pacific but also by widespread biases in the other tropical oceans.

Further support for the view that the warming trend in the satellite data is mostly false comes from Fig. 3. It shows, for January 1982–December 1988 and the Northern Hemisphere, seasonally averaged (1) UKMO SST anomalies, (2) UKMO night marine-air-temperature (NMAT) anomalies and (3) air-temperature anomalies on land^{8,9}. These data are all referred to a 1951–80 average. The correlation between seasonal SST and NMAT data sets over this period is very high ($r=0.92$); that between the land air and ocean is less, as the land data show more interseasonal variability ($r=0.54$ with SST and $r=0.62$ with NMAT). Nevertheless, neither the land nor the marine data show any significant trend over the period 1982–88. Indeed, two El Niño/Southern Oscillation events dominated the record so completely that calculation of a trend over this period is almost meaningless.

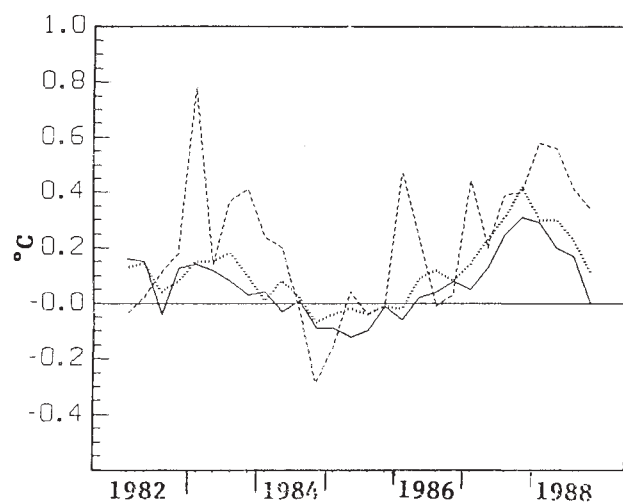


FIG. 3 Seasonal anomalies for the Northern Hemisphere referred to a 1951–80 climatology for January–March 1982 to October–December 1988: Solid line, UKMO sea surface temperature; dotted line, UKMO night marine air temperature; dashed line, land air temperature^{8,9}.

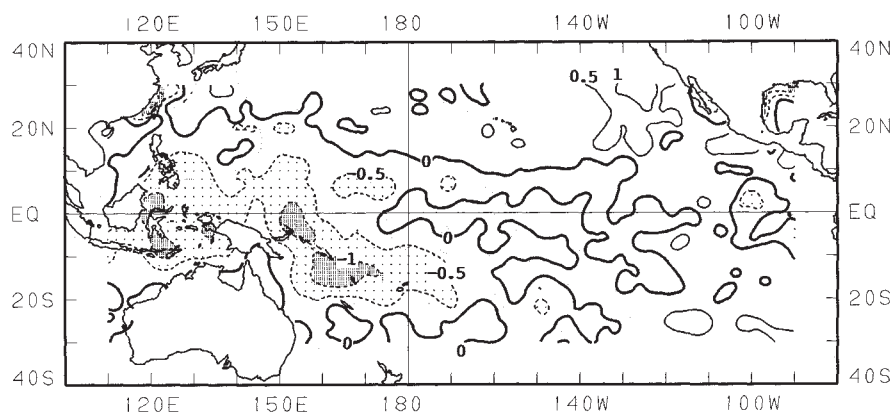


FIG. 4 Mean difference between daytime and night-time satellite SST values over the tropical Pacific for February 1989. The contour interval is 0.5°C; solid contours indicate positive differences and dashed lines negative differences. The heavy solid line is the zero-difference contour. Dotted regions indicate differences between -0.5°C and -1°C. Stippled regions indicate differences more negative than -1°C.

We are much less certain about the adequacy of *in situ* data for the Southern Hemisphere because of its poor coverage. Southern Hemisphere satellite data may not always be completely adequate, however, even after all possible influence from El Chichón ceased. In Fig. 4 we show satellite day-minus-night differences for February 1989, a more recent period than that covered by Strong. In the western tropical Pacific, there is a large region where daytime satellite SSTs are more than 0.5°C colder than corresponding night-time values. This pattern had persisted since the new NOAA-11 satellite became operational in November 1988, but tropical daytime SSTs could not be colder than night-time SSTs for such a long period. Comparisons of satellite retrievals with drifting-buoy SST measurements in this region since November 1988 confirm that daytime satellite temperatures are lower than the buoy temperatures. Such problems must be corrected before satellite data can be used on their own to estimate climate trends.

Accurate global SST data from satellites are urgently needed,

especially in the Southern Hemisphere, to help provide trustworthy analyses of the expected future global surface warming. For the recent past, the best outcome would be that satellite SST data available since the 1970s could be corrected (retrospectively) for their biases, blended with (corrected) *in situ* SST data and combined with surface-land and sea-ice analyses. □

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1. Strong, A. E. *Nature* **338**, 642-645 (1989).
2. Reynolds, R. W. *J. Clim.* **1**, 75-86 (1988).
3. Parker, D. E. & Folland, C. K. *Recent Climate Change*, 41-50 (Belhaven, New York, 1988).
4. Bottomley, M., Folland, C. K., Hsiung, J., Newell, R. E. & Parker, D. E. *Global Ocean Surface Temperature Atlas* (Massachusetts Institute of Technology Press, in the press).
5. Folland, C. K., Parker, D. E. & Kates, F. E. *Nature* **310**, 670-673 (1984).
6. Robock, A. *Nature* **341**, 695 (1989).
7. Oort, A. H., Pan, Y. H., Reynolds, R. W. & Ropelewski, C. F. *Clim. Dyn.* **2**, 29-38 (1987).
8. Jones, P. D. *et al. J. Clim. appl. Met.* **25**, 161-179 (1986).
9. Jones, P. D. *J. Clim.* **1**, 654-660 (1988).

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Beryllium in marine pore waters: geochemical and geochronological implications

D. L. Bourlès*, G. Klinkhammer, A. C. Campbell, C. I. Measures, E. T. Brown & J. M. Edmond

Department of Earth, Atmospheric and Planetary Sciences, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA

DIAGENETIC remobilization and hydrothermal inputs have been invoked as possible explanations for the discrepancy between the theoretical and measured temporal decrease of the non-detrital $^{10}\text{Be}/^{9}\text{Be}$ ratio observed in sediments¹. Remobilization is also consistent with water-column profiles of beryllium, which indicate a significant benthic flux from sediments². To evaluate the impact of diagenetic recycling on the beryllium isotopic composition of marine waters and the effects of this process on the beryllium geochronometer, we have analysed the ^{9}Be content of interstitial waters from different environments. Here we report the first pore-water profiles for ^{9}Be in oxic and suboxic pore waters as well as some preliminary results from hydrothermal sediments collected in the Guaymas Basin. Our results suggest that the use of the beryllium isotopes as a geochronometer is not affected either by sedimentary diagenesis at oxic and suboxic sites or by input of hydrothermally derived ^{9}Be more than a few kilometres from vent sites.

* Present address: Centre de Spectrométrie Nucléaire et de Spectrométrie de Masse, Batiment 108, 91405 Campus Orsay, France.

Oxic pore waters were collected at MANOP site S in the central equatorial Pacific; suboxic pore-water samples were collected at the Patton Escarpment (Southern California Borderland)³. Subcores taken with plastic core liners from box cores were sectioned and centrifuged at *in situ* temperatures and, at the suboxic site, under nitrogen. Pore water thus obtained was immediately filtered and acidified.

Interstitial waters from Guaymas Basin were obtained during dives of the submersible *Alvin* in January 1982. Core 1172 is from the Southern Vent Field immediately adjacent to an active 20-m-high hydrothermal mound, and core 1174 was taken in the vicinity of East Hill near hydrothermal deposits⁴. They were obtained with plastic *Alvin* core tubes. The sediment was extruded, sectioned and squeezed in an acid-cleaned plastic pore-water press. The fluids were immediately filtered and sealed in glass ampoules.

Beryllium analyses were made by gas chromatography with electron-capture detection of its 1,1,1-trifluoro-2,4-pentanedione derivative. The details of the method have been presented in ref. 5. Aliquots ranging from 10 to 30 ml were required for these analyses.

Beryllium concentration-depth profiles for the oxic and sub-oxic sites are presented in Fig. 1 along with interstitial Mn and Si data. The beryllium concentrations near the interface are a factor of 2.5 (MANOP site S) and 3.3 (Patton Escarpment) higher than the average North Pacific bottom water (29.4 ± 1.7 pM, refs 2 and 7). This, coupled with the observed increase in concentration with depth, implies a flux of beryllium out of the sediments.

We estimated steady-state fluxes to bottom waters for each location assuming molecular Fick's-law diffusion and a linear concentration gradient across the interface. We estimated an effective bulk sediment diffusion coefficient of $1.7 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$

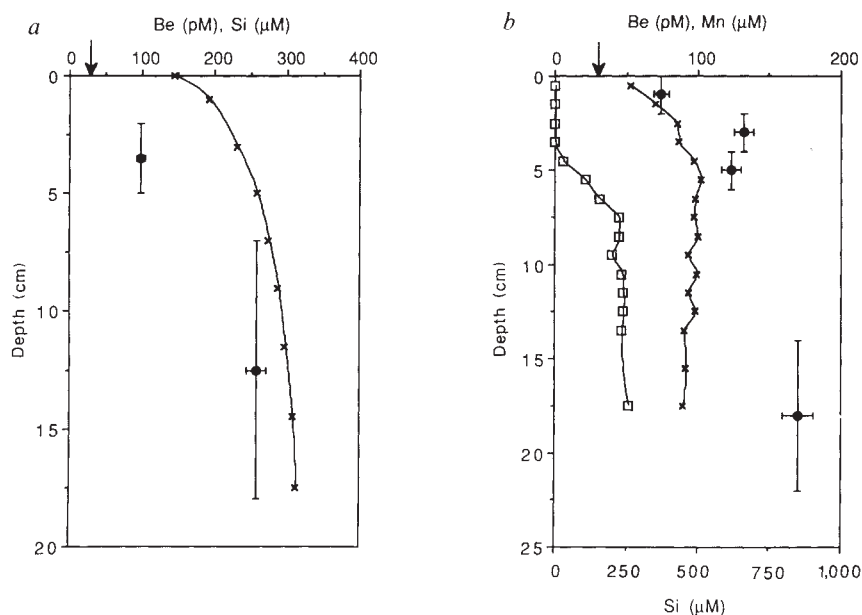


FIG. 1 Pore-water ^9Be (filled circles), Mn (open squares) and Si (crosses) as a function of depth. *a*, MANOP site S (oxic environment). This site, selected to represent typical provinces of siliceous sedimentation, is located at 11°N , 140°W with a water depth of 4,910 m. Oxygen was present throughout the core and no appreciable dissolved Mn was detected. *b*, Patton Escarpment site BC-10 (suboxic environment). This site is located at 32.5°N , 120°W with a water depth of 3,800 m. Mn and Si data are from a nearby core, PSC-10 (ref. 6). The arrows indicate Be bottom-water value.

by applying the Stokes-Einstein correction for temperature to the value calculated⁸ for Be^{2+} and using a porosity of 90%^{3,9} and an estimated tortuosity factor of 0.7. The concentration gradients are $18.5 \pm 0.7 \text{ pM cm}^{-1}$ at MANOP site S and $35.1 \pm 6.3 \text{ pM cm}^{-1}$ at Patton Escarpment, which yield lower limits for total flux of $1.0 \text{ pmol cm}^{-2} \text{ yr}^{-1}$ (Site S) and $1.9 \text{ pmol cm}^{-2} \text{ yr}^{-1}$ (Patton). Within reasonable uncertainties, these fluxes are consistent with the estimate² derived from the distribution of beryllium in the water column of the central North Pacific ($1.5 \text{ pmol cm}^{-2} \text{ yr}^{-1}$). These values are negligible compared with the non-detrital Be flux to the sediments.

At MANOP Site S and Patton Escarpment the correlation coefficients between pore-water Be and pore-water Si ($r = 0.998$ and 0.905 respectively, Fig. 1) suggest that the Be released to pore water is associated with the silica being dissolved in these sediments. At MANOP site S, the Be/Si mole ratio in the pore water calculated from the concentration gradients of both elements in the upper 3.5 cm is 715×10^{-9} . Averaging the Si concentrations over the depth interval 7–18 cm, we calculate a Be/Si ratio of 782×10^{-9} . At Patton Escarpment, the Be/Si ratios estimated the same way are 398×10^{-9} and 376×10^{-9} in the upper 3 cm and at 14–22 cm respectively. These values are con-

sistent with a biogenic opaline source for Be, given the uncertainties in the Be/Si ratio of amorphous biogenic silica (Table 1). They indicate that the depth-concentration profiles of both elements are linked to the solubility of amorphous silica¹³ which may be controlled by clay mineral-aqueous silica equilibria¹⁵.

We note that the highest levels of ^9Be in pore water measured in this study (258 pM) represents less than 0.02% of the beryllium present in the non-detrital phase of the associated sediments¹. As the $^{10}\text{Be}/^9\text{Be}$ ratio in opal is also nearly equal to that in the hydroxylamine-leachable phase¹ of the sediments, it seems that remobilization of beryllium isotopes during sedimentary diagenesis does not affect the $^{10}\text{Be}/^9\text{Be}$ ratio of the non-detrital phase and therefore does not affect the use of Be isotopes as a geochronometer.

The pore-water data from the Guaymas Basin are presented in Fig. 2 with the associated dissolved-Mn profile. Not only are the shapes of the profiles different from those observed in pelagic and hemipelagic pore waters but the concentrations are also one to two orders of magnitude higher and show more variability. In fact, the ^9Be concentration measured near the sediment-water interface in core 1172-2 is at the low end of the range for Be in hydrothermal fluids^{15,16}.

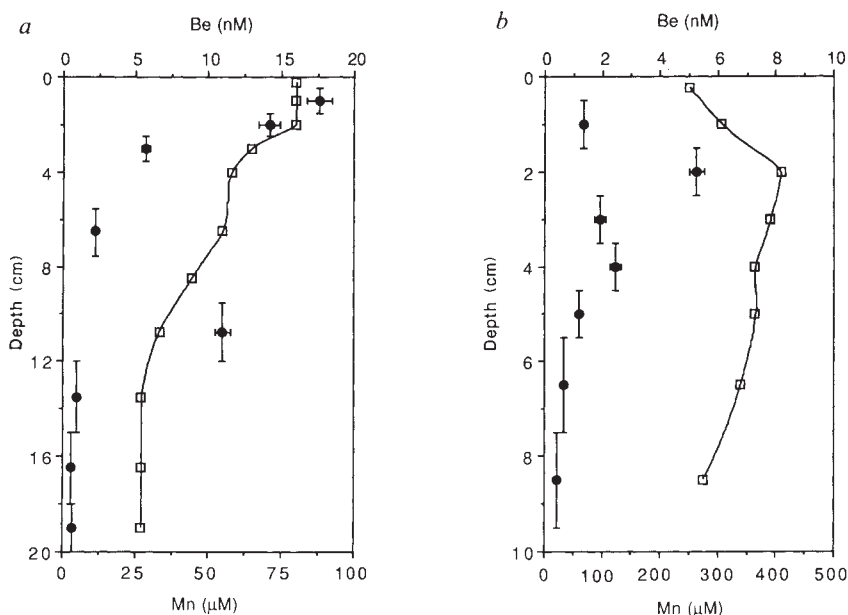


FIG. 2 Pore-water ^9Be (filled circles) and Mn (open squares) as a function of depth. *a*, Core 1172-2. *b*, Core 1174-3. Core 1174-3 had not undergone any hydrothermal alteration, whereas core 1172-2 showed signs of previous hydrothermal alteration in the solids, but the interstitial waters indicated that no advection of hydrothermal fluids was occurring when it was collected⁴.

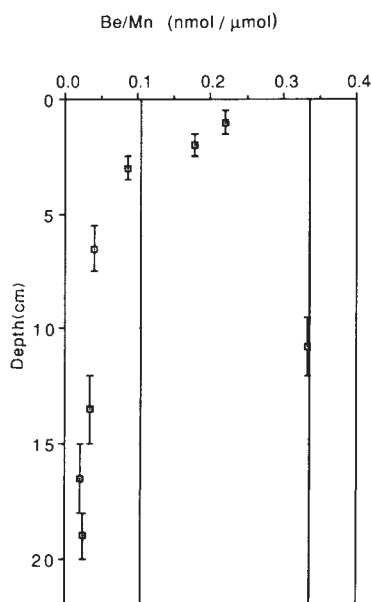


FIG. 3 Pore-water Be/Mn ratios from Guaymas Basin as a function of depth. Vertical lines represent the limits of the range of Be/Mn ratios in hydrothermal end members from Guaymas Basin.

Be and Mn seem to be related in the pore waters of this hydrothermally influenced environment—the correlation coefficients for cores 1172-2 and 1174-3 are 0.757 and 0.746 respectively. As shown in Fig. 3, several samples from core 1172-2 have Be/Mn ratios well within the range of Be/Mn ratios in hydrothermal fluids from the Guaymas Basin¹⁵: $(219 \pm 115) \times 10^{-6}$.

These observations suggest a near quantitative co-precipitation of beryllium with the hydrothermal precipitates formed near the vent during the mixing of reduced hydrothermal fluids with oxidizing sea water⁴. A pair of water-column profiles of Be in the Guaymas Basin¹⁷, taken directly over an active vent (12 CTD) and about 1 km away from the region of active vents (11 CTD), also implies that the efficiency with which Be is scavenged near vents restricts the dispersal of hydrothermally derived ⁹Be to within a few kilometres from the source. The general decrease of the pore-water Be/Mn ratio with depth in the sediments at Guaymas indicates that the beryllium solubilized during diagenesis of these precipitates is partitioned onto sediments more efficiently than the manganese. Although the interstitial-water chemistry of core 1172-2 indicates no advection of hydrothermal fluids⁴, the maximum observed at 11 cm may be a result of enhanced hydrothermal influence. Thus, lateral and vertical fluctuations in the Be/Mn ratio may reflect the spatial and temporal variability of hydrothermal inputs for surficial sediments.

In a study¹ of the potential use of the non-detrital ¹⁰Be/⁹Be ratio for dating marine sediments, Bourlès *et al.* invoked

TABLE 1 Be/Si mole ratios for different marine phases

	Alumino-silicates*	Hydrothermal water		Organic matter‡
		Guaymas Basin	Opal†	
Be/Si	28×10^{-6}	$(2.85 \pm 1.87) \times 10^{-6}$	$(0.75 \pm 0.08) \times 10^{-6}$	≈ 5

* Assuming average concentrations of 2.5 p.p.m. for Be and of 60% SiO₂ in the aluminosilicates.

† From data of ref. 1 by assuming that the studied sediment from Central Equatorial Pacific (RC12-65) is 50% opal⁹.

‡ This estimate is based on concentrations measured in the organic fraction of plankton^{11,12}.

sedimentary diagenesis and the input of hydrothermally derived ⁹Be, among other possibilities, to explain the observed deviation from the expected decay. This work suggests that these processes do not influence the Be isotopic composition of deep-sea sediments. Nevertheless, beryllium released to pore fluids, probably during the dissolution of amorphous biogenic silica, results in fluxes out of the sediments at the oxic and suboxic sites that are large enough to support the near-bottom increases observed in the water column. Beryllium scavenged by hydrothermal precipitates in the vicinity of submarine hydrothermal vents is also released to pore water by diagenetic reduction in the sediment column. The influence of hydrothermally derived Be is, however, limited to within a few kilometres of the source because of its rapid removal. □

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- Bourlès, D. L., Raisbeck, G. M. & Yiou, F. *Geochim. cosmochim. Acta* **53**, 443–452 (1989).
- Measures, C. I. & Edmond, J. M. *Nature* **297**, 51–53 (1982).
- Reimers, C. E. & Smith, K. L. Jr *Limnol. Oceanogr.* **31**, 305–318 (1986).
- Campbell, A. C., Gieskes, J. M., Lupton, J. E. & Lonsdale, P. F. *Geochim. cosmochim. Acta* **52**, 345–357 (1988).
- Measures, C. I. & Edmond, J. M. *Anal. Chem.* **58**, 2065–2069 (1986).
- Shaw, T. J. thesis, Univ. of California, San Diego (1988).
- Kusakabe, M. *et al. Earth planet. Sci. Lett.* **82**, 231–240 (1987).
- Li, Y. H. & Gregory, S. *Geochim. cosmochim. Acta* **38**, 703–714 (1974).
- Buchholtz, M. thesis, Columbia Univ. (1987).
- Broecker, W. S. & Peng, T. H. *Tracers in the Sea* (Lamont-Doherty Geological Observatory, New York, 1982).
- Bourlès, D. L. *et al. Nucl. Instrum. Meth.* **233**, 365–370 (1985).
- Martin, J. H. & Knauer, G. A. *Geochim. cosmochim. Acta* **37**, 1639–1653 (1973).
- Alexander, G. B., Heston, W. M. & Iler, R. K. *J. phys. Chem.* **58**, 453–455 (1954).
- Bischoff, J. L. & Ku, T. L. *J. Sedim. Petrology* **40**, 960–972 (1970).
- Von Damm, K. L., Edmond, J. M., Measures, C. I. & Grant, B. *Geochim. cosmochim. Acta* **49**, 2221–2237 (1985).
- Bourlès, D. L. *et al. Eos* **70**, 494 (1989).
- Campbell, A. C. thesis, Univ. California, San Diego (1985).

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A new self-organizing mechanism for deep-focus earthquakes

H. W. Green II & P. C. Burnley

Department of Geology, University of California, Davis, California 95616, USA

THE mechanism of deep-focus earthquakes has been a puzzle since their discovery almost 70 years ago^{1,2}, because brittle fracture and frictional sliding at depths in excess of 100–200 km would require unrealistic rock strengths^{3,4}. Rock strength does increase with pressure, but a few hundred MPa (equivalent to 10–20 km depth) suffices to inhibit most fracture, and elevated temperature activates ductile mechanisms that operate at stresses less than the fracture strength. A range of mechanisms has been proposed for deep earthquakes, including plastic instabilities^{5–7}, shear-induced melting^{8–11} and instabilities accompanying recrystallization^{12,13} or polymorphic phase transformation^{14–23}. Each of these proposed mechanisms has exhibited certain inherent weaknesses (see Kirby²² for review and discussion). Here we report experimental observations of high-pressure faulting of metastable Mg₂GeO₄ olivine as it undergoes incipient transformation to a spinel^{24,25} structure. We present a model in which this faulting arises from the generation, propagation and linking-up of spinel-filled anticracks²⁶. When applied to the olivine → spinel transformation in the Earth's mantle, the anticrack model²⁷ satisfactorily accounts for the similarities and differences between shallow and deep earthquakes and removes the problems associated with frictional sliding.

Most previous models of deep earthquakes postulate *a priori* the existence of a planar structure (physical or thermal) that is inclined to the principal axes of stress and which evolves into the fault. By contrast, the microstructures of our specimens suggest a new, self-organizing mechanism that is intimately

connected to the transformation process and which exhibits striking analogy to the Griffith theory of brittle fracture.

Direct experimental evidence that may bear on the origin of deep-focus earthquakes has been meagre^{22,23}. Both previous studies proposed rapidly running phase transformations to explain their observations but neither involved phases expected to be present in the Earth's mantle. The process producing deep earthquakes proposed in both studies involves similar phenomena. In particular, Kirby²² proposed that initiation of transformation to a denser phase in a planar zone under shear stress could stimulate runaway transformation in that plane, leading to faulting. The faulting that we have found to develop during the olivine $\rightarrow$ spinel transition is directly relevant to the Earth's interior and confirms the concept of a shearing instability triggered by phase transformation^{18,22}. Moreover, our experiments have two important and unforeseen characteristics that provide insight into the physical mechanism responsible for the instability. First, faulting occurs only in a narrow temperature interval^{24,25} where incoherent nucleation and growth becomes just possible on the timescale of the experiments^{25,28}; the material is ductile both above and below this region. Second, under the conditions of instability, lens-shaped regions of very fine-grained spinel develop at high angles to the maximum compressive stress, σ_1 ; this morphology is distinctly different from that which develops when the transformation occurs under hydrostatic pressure. Based on these observations we propose a simple mechanism that makes the existence of deep earthquakes and their distribution not only plausible but expected.

The conditions of our experiments fall into three categories (Fig. 1). At low temperatures, the specimens are strong and ductile; martensitic (shear-induced) nucleation of spinel is possible (under sufficiently high stresses) but growth of the nuclei is kinetically inhibited²⁸ and faulting does not occur. At high temperatures, abundant incoherent nucleation and rapid growth of spinel are observed^{25,29} and the specimens are weak and ductile²⁵; again, the specimens show no instability. The intervening narrow temperature interval marks the onset of diffusion-assisted growth of martensitic nuclei²⁸ and also the beginnings of incoherent nucleation²⁵; within this window, faulting occurs in every experiment except those purposely stopped early. Increasing the strain rate to 10^{-3} s^{-1} shifts the window to higher temperatures²⁵.

Optical and electron microscopy of specimens deformed within the window of instability show the following: (1) lenses of spinel develop with a very strong preferred orientation at angles to σ_1 approaching 90° (Fig. 2a); (2) spinel within the lenses is invariably very fine-grained (Fig. 2b); (3) spinel lenses sometimes develop in en echelon arrays that lie in orientations

of high shear stress (Fig. 2c); (4) unfaulted specimens, including those in experiments stopped just after onset of the instability (but before faulting), show no growth of spinel in planes of high shear stress; (5) faults contain fine-grained spinel²⁵; (6) en echelon microfaults occur with stepping in the opposite sense to that commonly observed in brittle faulting (in one experiment, small spinel-lined faults are linked by push-togethers exhibiting enhanced transformation in the form of spinel microlenses, whereas in brittle behavior, faults are linked by pull-aparts³⁰).

The crack-like morphology of the spinel lenses coupled with their preferred orientation normal to σ_1 reminds us of the anticrack model²⁷ of stylolites (solution surfaces). The term 'anticrack' was devised because anticracks are identical to tension (mode I) cracks except that the algebraic signs of the displacements and stresses around them are reversed. Consider a solid with a small planar flaw that is acted upon by a macroscopic tensile stress σ_3 (Fig. 3a). Under the stress, the flaw becomes an open mode I crack with zero stress across it and high tensile stress at its tips. Similarly, if one imagines a lenticular void (of the dimensions of the crack in Fig. 3a) within an unstressed solid and applies a far-field compressive stress, σ_1 , just sufficient to close the void, one obtains an 'open' mode I anticrack with zero stress across it and high compressive stress at its tips (Fig. 3b). If the crack is not normal to σ_3 , its tips deviate during growth so as to approach that orientation^{31,32}. Similarly, if an anticrack is not normal to σ_1 , it will become so during growth (by dissolution in the case of stylolites). This is the model of stylolites in ref. 27, which captures the essence of their shape, orientation and geological setting.

Here we extend the analogy between cracks and anticracks to encompass filled versions of the two. We consider a small lenticular volume within a solid that undergoes a phase transformation. If the transformation involves a volume increase, the resulting stress distribution will be as shown in Fig. 3c. Similarly, if the phase change leads to densification (as does the olivine $\rightarrow$ spinel transformation), the stress distribution will be as shown in Fig. 3d. Superposition of a tensile stress on Fig. 3c or a compressive stress on Fig. 3d will enhance the effects at the tips while relieving the stresses across the surfaces of the lens, leading to stress distributions similar to those of Figs 3a and b. In all cases, the magnitude of the stress concentrations is controlled primarily by the curvature at the tips; the more pointed the tip, the higher the stress concentration. The tendency for the tips of a propagating filled anticrack to rotate toward the plane normal to an applied compressive stress is the same as for an empty anticrack. In this case it does so by enhanced transformation to the stable phase(s) as a result of the high compressive stresses at the tips. The rheology of the filling material will become critical should a shearing instability develop. The orientation and the striking crack-like morphology of the spinel lenses in our specimens lead us to conclude that they are mode I anticracks with these characteristics.

Under fluid-absent conditions, tensile microcracks are closed by application of pressure, and tensile (mode I) failure is inhibited; brittle failure occurs by generation of large numbers of mode I microcracks and development of an instability which leads to shear (mode II) fracture. The fundamental process by which the mode I (Griffith) microcracks propagate and link together to produce faulting is poorly understood because mode II cracks do not propagate in their own plane^{31,32}. Petit and Barquin³² concluded that "mode II cannot exist as an elementary (primary) fracture mechanism but can only be a macroscopic fracture phenomenon which must necessarily involve tensile (mode I) microcrack formation". This description of brittle failure is remarkably similar to our observations in that we see abundant mode I anticracks but no evidence of shear failure in advance of macroscopic faulting. We therefore propose that shear failure in our experiments occurs by anticracking, with the generation and linking of mode I features being analogous to similar processes occurring in normal brittle failure. The

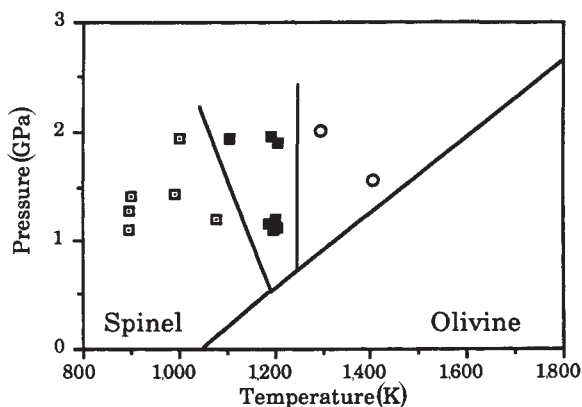


FIG. 1 Conditions of experiments conducted at strain rates of 10^{-4} – 10^{-5} s^{-1} on metastable Mg_2GeO_4 olivine. Open squares represent specimens that were strong and ductile, open circles, specimens that were weak and ductile, and closed squares, specimens that failed. See text for discussion. Phase boundary after ref. 42.

instability is not a friction-related phenomenon, however, because the fine-grained nature of the spinel produced under these conditions renders it superplastic³³. As a consequence, the mechanism is not inhibited by high pressure.

For application of this mechanism to deep faulting within the Earth, we first must consider the differences between the germanate system of our experiments and the silicate system of the mantle, and the distribution and characteristics of deep earthquakes. Germanates have been shown to be faithful analogues for silicates, especially with respect to the olivine $\rightarrow$ spinel transition^{28,34}. Our experiments on Mg_2GeO_4 relate only to the $\alpha \rightarrow \gamma$ transformation, however, whereas in the Earth the transformation in $(\text{Mg}, \text{Fe})_2\text{SiO}_4$ olivine will be $\alpha \rightarrow \beta$ or $\alpha \rightarrow \gamma$ depending on the conditions at which the phase transition occurs³⁵. As anticracks are driven only by volume reduction under stress and because the β phase also is more dense than olivine, our mechanism should apply equally to either transformation.

Figure 4a shows the depth distribution of earthquake frequency in descending slabs of lithosphere beneath island arcs³⁶ and Fig. 4b shows a model of the temperature distribution expected in such slabs along with the accompanying perturbation of the equilibrium boundary for the olivine-spinel transfor-

mation³⁷. Our results indicate that in the central, colder parts of the slab the transformation is kinetically inhibited until it reaches a set of critical conditions at which nucleation and growth of spinel become possible. The critical conditions depend on the presence of a nonhydrostatic stress and on the temperature, the timescale on which the stresses are applied, the degree of overstepping of the phase boundary (and hence the pressure) and the amount and rate of plastic deformation imposed on the metastable olivine. The onset of the transformation is controlled by kinetic factors and therefore temperature and time are the most important parameters, just as they are in our experiments. Therefore, for simplicity of presentation, we will discuss the possibility of earthquakes in terms of a critical temperature at which nucleation and growth become just possible on the timescale involved. Neglecting any depth dependence of the critical temperature T_c we see that if, for natural olivine, $T_c = 1,300$ K for the hypothetical slab in Fig. 4b, the olivine $\rightarrow$ spinel transformation could be responsible for all deep earthquakes. Alternatively, if $T_c = 1,200$ K, olivine $\rightarrow$ spinel

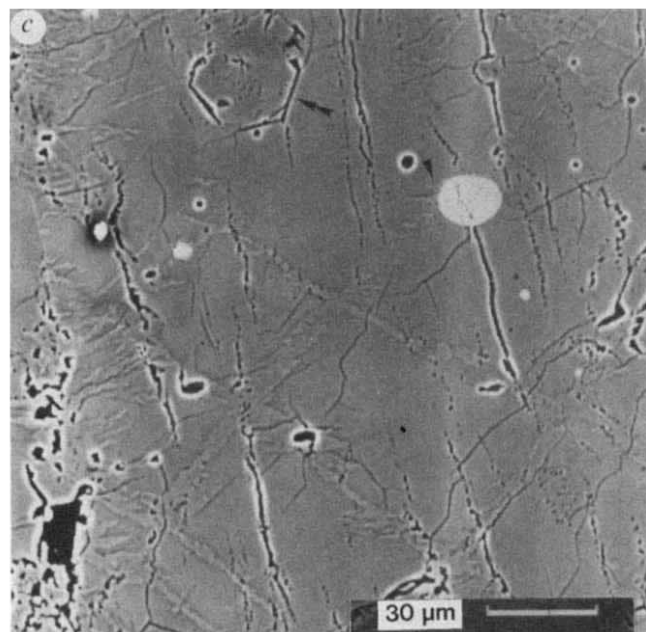
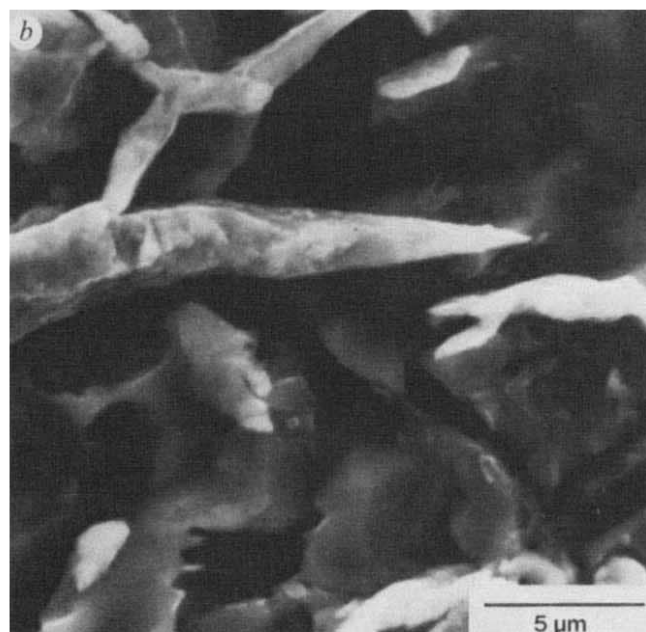
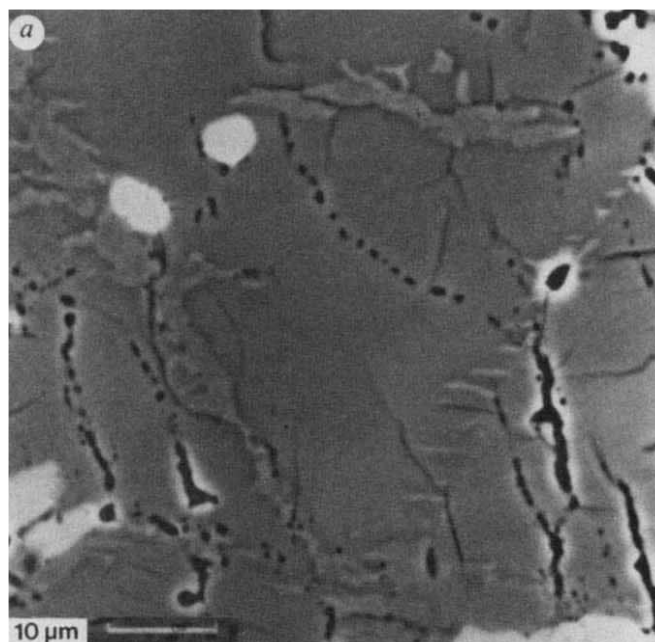


FIG. 2 Microstructures of faulted specimens; the maximum compressive stress σ_1 is vertical in all micrographs. *a*, Backscattered-electron (BSE) scanning electron micrograph of a polished specimen showing transformation of $\alpha\text{-Mg}_2\text{GeO}_4$ (olivine) to $\gamma\text{-Mg}_2\text{GeO}_4$ (spinel). The spinel phase (light grey) nucleates on the grain boundaries between olivine crystals (dark grey) and on pyroxenes (white); it grows as thin lenses, the long axes of which exhibit a strong preferred orientation normal to σ_1 . Black regions (that often appear as dotted lines) are quench cracks that have been partially filled by the syton polishing process. *b*, Secondary-electron scanning electron micrograph of a specimen etched in HCl to enhance visibility of spinel. The surface mottling of the spinel lenses (white) is due to dissolution at grain boundaries and shows that they are composed of equant, very fine-grained ($<1\ \mu\text{m}$) crystals. *c*, BSE micrograph of a polished specimen showing intersecting arrays of en echelon spinel lenses within a single crystal of olivine and abundant growth of lenses on a grain boundary (left). Lenses also can be seen growing off of a pyroxene inclusion (single arrowhead). The large, equant, faceted crystal near the top (double arrowheads) is a cube-modified octahedron of spinel with the topotaxy expected for the martensitic nucleation mechanism²⁸. The crystallographic control of the habit of crystals nucleated by coherent mechanism and the nucleation of lenses on inclusions and grain boundaries suggest that the lenses are the result of the incoherent mechanism.

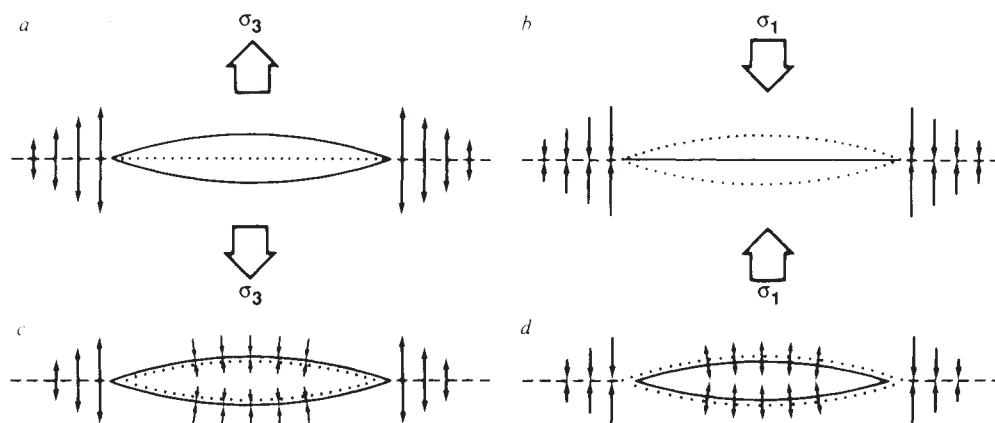


FIG. 3 Comparison of mode I cracks and anticracks. *a*, A small planar flaw in a solid across which cohesion has been lost (dotted line) is pulled open by a remote tensile stress, σ_3 , yielding a lens-shaped void (crack) with tensile stress concentration at its tips. *b*, A small lenticular void (dotted) in a solid is pushed closed by a remote compressive stress, σ_1 , yielding a compressive stress concentration at its tips. The stress distribution is identical that in *a* except that the algebraic signs have been reversed. This is an empty anticrack. *c*, Local phase transformation of a lens-shaped volume (dotted) in an unstressed solid that expands on transformation. The enlarged lens has compressive stresses across its surfaces and tensile

stresses at its tips. *d*, Phase transformation to a denser phase in a lens-shaped volume (initial dimensions dotted) leads to a stress distribution the inverse of that found in *c*. This is a filled anticrack. For the application of interest in this paper, we superpose onto the stresses shown in *d* a far-field nonhydrostatic stress for which the mean stress $(\sigma_1 + \sigma_2 + \sigma_3)/3$ is of the order of 1–2 GPa for our experiments and 8–18 GPa for the Earth. We arrive at a system with a lens of spinel embedded in olivine with the (compressive) normal stress across the lens less than the macroscopic normal stress in that direction and with enhanced compression at the tips.

would be exhausted as a mechanism by ~ 600 km and another transformation such as spinel $\rightarrow$ perovskite + magnesiowüstite) would be needed for deeper earthquakes. Although we do not yet know the T_c for natural olivine under mantle conditions, these temperatures are within the range proposed³⁸ for the onset of transformation in mantle olivine. It is therefore clear that the known thermal structure of descending slabs makes it unavoidable that material within subducting lithosphere must encounter conditions appropriate for the kind of instability that we propose, unless the stresses are too low for faulting.

Although it is not yet clear how incoherent nucleation and growth can operate at rates sufficient to generate faulting, our results suggest that anticracks and the shear instability are unrelated to the martensitic nucleation mechanism (Fig. 2c). We postulate that the instability follows development of a critical density and/or distribution of mode I anticracks and incipient loss of coherence of the olivine framework; catastrophic failure follows when, during the first stages of collapse, the heat generated locally by transformation at points of stress concentration causes the next increment of reaction to be faster, leading to runaway of transformation in a narrow zone. It is encouraging that deep earthquakes seem to be relatively slow — “The times and distances to rupture subevents indicate that the failure process propagates with a speed which is less than the S wave speed”³⁹.

Our model also predicts that faulting should not occur repeatedly in the same material because the transformation process is intimately involved in the faulting. That is, repeated stick-slip is not to be expected because after faulting the fault zone is lined with superplastic spinel that should be ductile. That is what we see in our experiments²⁵ and it is also consistent with the seismic data; the aftershock distribution of deep-focus events is incompatible with simple slip on a single fault³⁹. After some time, the spinel within the fault zone should coarsen, lose its superplastic properties, and may become stronger than olivine, preparing the way for further faulting nearby.

Unless a fluid is produced, this, or any other, phase-transformation mechanism cannot explain earthquakes at depths < 250 –300 km because there are no known appropriate solid-solid transformations that could occur in an interconnected phase of peridotite, basalt or eclogite. The depth distribution shown in Fig. 4a suggests⁴⁰ a superposition of two distinct varieties of seismic activity. We are tempted to decompose the data into two distributions and attribute the shallow component to failure

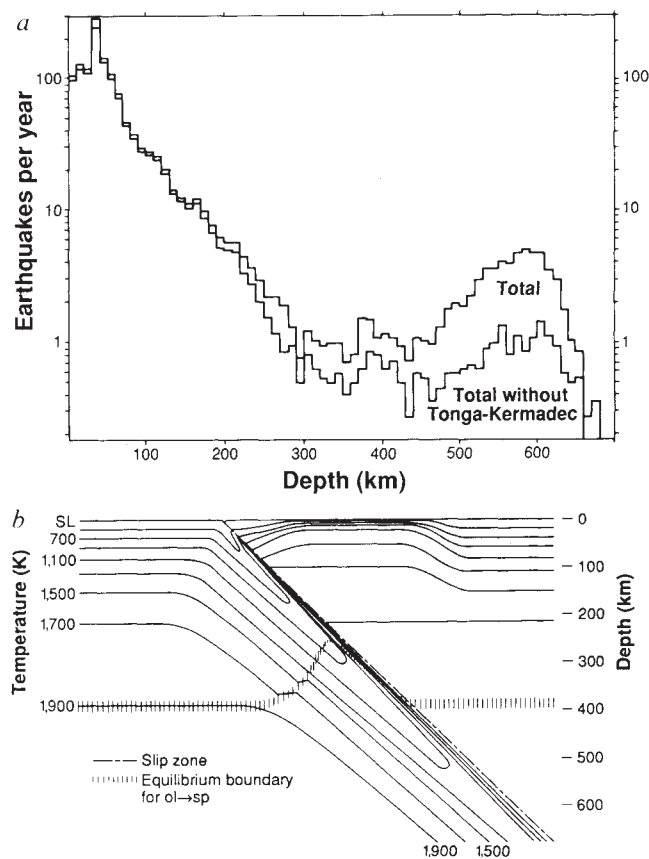


FIG. 4 *a*, Depth dependence of earthquake frequency for moderate and large earthquakes during the period January 1964–February 1986 for the entire Earth (Total) and for the Earth except for the Tonga and Kermadec subduction zones (after ref. 36). Note exponential falloff to 300 km, marked increase again to 550–600 km and abrupt cutoff below 650 km. *b*, Thermal model of descending slab of lithosphere (after ref. 37) showing shape of isotherms and distortion of equilibrium olivine-spinel phase boundary. Temperature contours $\geq 1,500$ K are depicted assuming that the transformation occurs (aseismically) with negligible overstepping of the phase boundary. For lower temperature, the contours pass through the equilibrium boundary unperturbed, implying that the transformation is delayed. Any volume of material in the cold interior of the slab must inevitably encounter the conditions of mechanical instability discussed in the text.

by cracking and the deep component to failure by anticracking. The exponential drop-off of the shallow component might be due to fluid-assisted cracking (Terzaghi effect)⁴¹ down to depths of perhaps 250 km; the similar rapid rise of the deep component is consistent with faulting by anticracking in response to increased overstepping of the olivine $\rightarrow$ spinel phase boundary at greater depths. Faulting finally falls off rapidly at depths >650 km as the spinel $\rightarrow$ perovskite + magnesiowüstite transformation is completed and no other transformations are available²². $\square$

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1. Turner, H. H. *Mon. Not. R. astr. Soc., Geophys. Suppl.* **1**, 1–13 (1922).
2. Wadati, K. *Geophys. Mag.* **1**, 162–202 (1928).
3. Jeffreys, H. *The Earth, Its Origin, History and Physical Constitution* 2nd Edn (Cambridge University Press, 1929).
4. Jeffreys, H. *Proc. R. Soc. Edinb.* **56**, 158–163 (1936).
5. Bridgman, P. W. *J. Geol.* **44**, 653–669 (1936).
6. Orowan, E. *Geol. Soc. Am. Mem.* **79**, 323–345 (1960).
7. Hobbs, B. E. & Ord, A. *J. geophys. Res.* **93**, 10,521–10,540 (1988).
8. Griggs, D. T. in *Nature of the Solid Earth* (ed. Robertson, E. C.) 361–384 (McGraw-Hill, New York, 1972).
9. Post, R. L. Jr *Tectonophysics* **42**, 75–110 (1977).
10. Griggs, D. T. in *Modern Physics for the Engineer* (ed. Ridenour, L. N.) 272–305 (McGraw-Hill, New York, 1954).
11. Griggs, D. T. & Handin, J. *Geol. Soc. Am. Mem.* **79**, 347–373 (1960).
12. Griggs, D. T. & Baker, D. W. in *Properties of Matter under Unusual Conditions*, (eds Mark, H. & Fernback, S.) (Wiley Interscience, New York 1969).
13. Ogawa, M. *J. geophys. Res.* **92**, 13,801–13,810 (1987).

14. Bridgman, P. W. *Am. J. Sci.* **243A**, 90–97 (1945).
15. Benioff, H. *Bull. seism. Soc. Am.* **53**, 893–903 (1963).
16. Evison, F. F. *Bull. seism. Soc. Am.* **53**, 873–891 (1963).
17. Evison, F. F. *Bull. seism. Soc. Am.* **57**, 9–25 (1967).
18. Vaisnys, J. R. & Pilbeam, C. C. *J. geophys. Res.* **81**, 985–988 (1976).
19. Sung, C. M. & Burns, R. G. *Tectonophysics* **31**, 1–32 (1976).
20. Liu, L. *Phys. Earth planet. Inter.* **32**, 226–240 (1983).
21. Hodder, A. P. W. *Phys. Earth planet. Inter.* **34**, 221–225 (1984).
22. Kirby, S. H. *J. geophys. Res.* **92**, 13,789–13,800 (1987).
23. Meade, C. & Jeanloz, R. *Nature* **339**, 616–618 (1989).
24. Burnley, P. C. & Green, H. W. *Eos* **70**, 473 (1989).
25. Burnley, P. C. & Green, H. W. *J. geophys. Res.* (submitted).
26. Green, H. W. & Burnley, P. C. *Eos* **70**, 473 (1989).
27. Fletcher, R. & Pollard, D. D. *Geology* **9**, 419–424 (1981).
28. Burnley, P. C. & Green, H. W. *Nature* **338**, 753–756 (1989).
29. Vaughan, P. J., Green, H. W. & Coe, R. S. *Tectonophysics* **108**, 299–322 (1984).
30. Rispoli, R. *Tectonophysics* **75**, T29–T36 (1981).
31. Paterson, M. S. *Experimental Rock Deformation—The Brittle Field* (Springer, Berlin, 1973).
32. Petit, J.-P. & Barquins, M. *Tectonics* **7**, 1243–1256 (1988).
33. Vaughan, P. J. & Coe, R. S. *J. geophys. Res.* **86**, 389–404 (1981).
34. Green, H. W. *Geophys. Res. Lett.* **11**, 817–820 (1984).
35. Bina, C. R. & Wood, B. J. *J. geophys. Res.* **92**, 4853–4866 (1987).
36. Frohlich, C. A. *Rev. Earth planet. Sci.* **17**, 227–254 (1989).
37. Turcotte, D. L. & Schubert, G. *Geodynamics* (Wiley, New York, 1982).
38. Sung, C.-M. in *High Pressure Science and Technology* Vol. 2 (eds Timmerhaus, K. D. & Barber, M. S.) 31–42 (Plenum, New York, 1979).
39. Willeman, R. J. & Frohlich, C. J. *geophys. Res.* **92**, 13,927–13,943 (1987).
40. Sykes, L. R. *J. geophys. Res.* **71**, 2981–3006 (1966).
41. Raleigh, C. B. & Paterson, M. S. *J. geophys. Res.* **70**, 3965–3985 (1965).
42. Ross, N. & Navrotsky, A. *Phys. Chem. Miner.* **14**, 473–481 (1987).

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Perception of multiple transparent planes in stereo vision

Daphna Weinshall

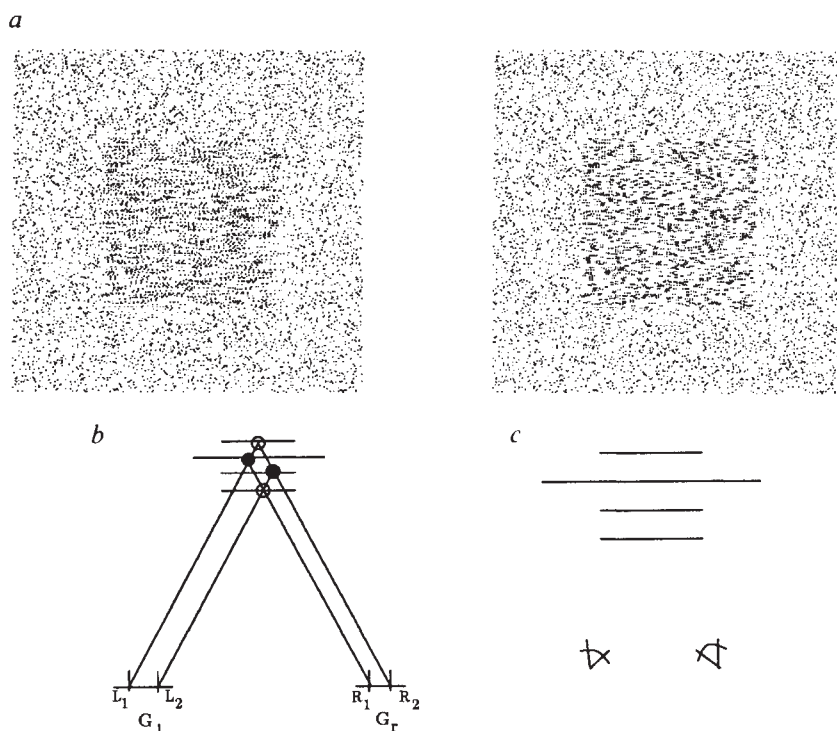
Center for Biological Information Processing, Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, E25-201, Cambridge, Massachusetts 02139, USA

INFORMATION about the distances of objects that we see can be obtained from the disparity in position of their images in our two eyes. This process entails solving the problem of stereo-matching,

which involves the determination of which points in the two images correspond to the same object. This is particularly problematic when features in the visual field are repeated, allowing more than one possible solution to the stereo-matching problem. Here I describe a situation in which a repetition of a random dot pattern leads to the perception of multiple transparent planes under some conditions, and of a single opaque plane under other conditions. This result shows that stereo-matching is not necessarily unique—a given point of the image in one eye may be matched simultaneously to more than one point in the other eye, each match defining a different depth plane. Current stereo-matching algorithms in computer vision do not account for these observations.

The stereoscopic matching of points in images involves a difficult computation, as shown in Fig. 1b. Here the two bars in the left image can be matched to the two bars in the right image

FIG. 1 *a*, An ambiguous stereogram with $G_r = 10^\circ$ and $G_l = 20^\circ$. After some time four planes emerge: two in front of the background, the background (all three transparent), and one behind the background. The deepest plane is often difficult to see; it helps to slightly diverge the eyes to capture it. Note the two bars ('nails') in the middle of the stereogram, which are usually seen on the two middle surfaces only, and which are hard to flip to the other surfaces. Because it takes some time for the impression to build, it is recommended to use a stereo viewer ~ 12.5 – 15.5 cm high. *b*, A graphic illustration of the projections of the two bars from the stereogram in *a*. The two pairs of matches that are mutually exclusive if matching is unique are separately marked by filled and hollow circles. This is also an illustration of the stimulus in the double nail illusion, where two nails are placed one behind the other with respect to the viewer. The resulting stereo pair looks like *b*, except that the ghost matches are in fact the correct ones. Humans, however, seem to prefer the order-preserving matches, and see an illusory percept of two nails side by side at the same depth³. *c*, The depth profile of the four possible matches of the stereogram in *a*. Braddick has used related stereograms (unpublished results; also see ref. 10) with effectively $G_r = 0$ (or $G_l = 0$).



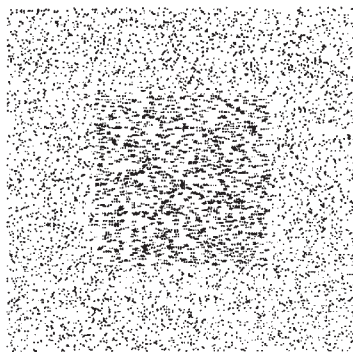
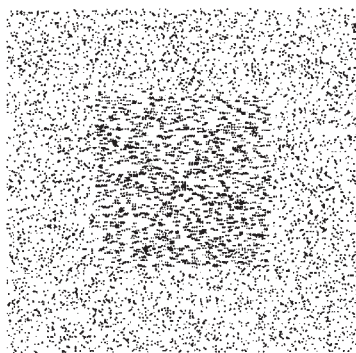
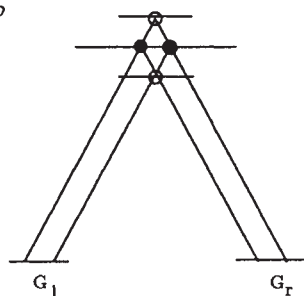
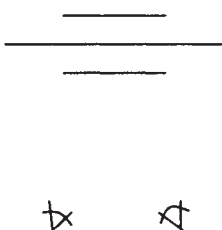
a*b**c*

FIG. 2 *a*, An ambiguous stereogram with $G_r = G_l = 10'$. Here only one opaque surface is seen, the background, and no vergence can help detect the other two surfaces (one above the background and one below it). *b*, A graphic illustration of the projections of two points from the stereogram in *a*. *c*, The depth profile of the three possible matches of the stereogram in *a*.

in four different ways. Two of these correspond to the matching of the left bar in the left image to the left bar in the right image, and the matching of the right bar in the left image to the right bar in the right image. These order-preserving matches are generally presumed to be correct^{1,2}. The other two matches are the 'ghost' or false matches. Humans seem to prefer the order-preserving matches even under experimental conditions where the ghost matches are in fact the correct ones (the double-nail illusion shown in Fig. 1*b*, see also ref. 3). The ambiguity of matching is higher with random dot stereograms, where each point has many false matches in the other image (see Fig. 1*a*). Matching algorithms are usually meant to resolve such

ambiguities and to obtain a unique correspondence (for example, see refs 4 and 5).

To test whether matching in human vision is always unique, I used an ambiguous random dot stereogram, which is an extension of the double-nail illusion. This was generated by first computing a random dot pattern, which was then copied twice in each image, with a different horizontal spacing of G_r minutes of arc in the right image and G_l minutes of arc in the left image (Figs 1*a* and 2*a*). The random pattern background was identical in both images. Observers who could fully fuse these stereograms saw four transparent surfaces corresponding to all the four possible matches shown in Fig. 1*b* (with the help of vergence

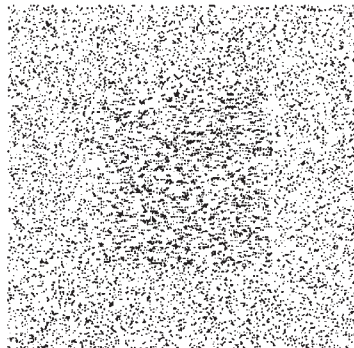
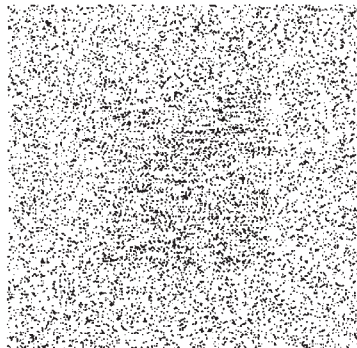
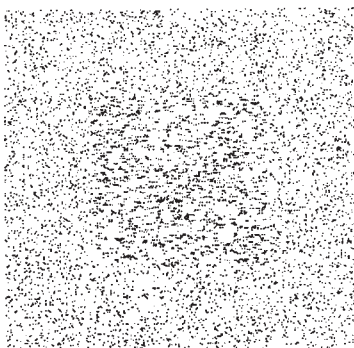
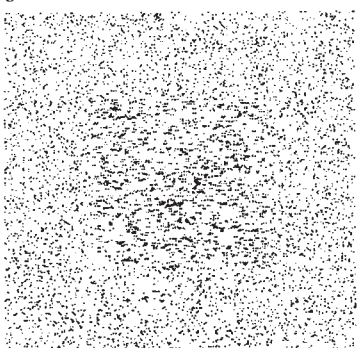
a*b*

FIG. 3 *a*, As in Fig. 1*a*, where the number of points in the deepest plane have been doubled with new unambiguous points. *b*, As in Fig. 2*a*, with an additional new uncorrelated plane at disparity $20'$, twice the disparity of the suppressed planes ($10'$ and $-10'$).

and memory; vergence is often needed in the perception of transparent stereograms⁶). These observers could judge the depth of the ghost surfaces correctly. This perception is unlike the perception of only two matches with single features (the double-nail illusion).

This result indicates that all sufficiently strong disparities give rise to the perception of distinct transparent surfaces. I define the strength of a given disparity at a given pixel to be the value of the correlation function between the two images (see Fig. 4 legend). A sufficient strength is a correlation value that is sufficiently above random. I call the corresponding disparity a 'solution'. To test the above hypothesis I used a special case of the stereogram that was used in the first experiment, in which $G_r = G_l$ (see Fig. 2). In this case there are three possible solutions (Fig. 4b). One might expect to see three transparent planes, by analogy with the first experiment. Surprisingly, only the strongest solution was seen, and this perception was quite robust.

Perhaps only solutions with approximately equal strength, that is, with comparable maxima in the correlation function, are detected, whereas weaker solutions are suppressed. To test this hypothesis I used the initial stereogram, in which $G_r \neq G_l$, but the amount of points in one plane was doubled by adding unambiguously matched points. All four solutions were still seen (Figs 3a and 4c). The other surfaces were visible even when the number of points in one surface was quadrupled.

The suppressed planes of Fig. 2a could be made to reappear for some observers. Either the number of points in each image was doubled by adding unmatched random dots, or correlated points were added to both images, forming a new plane (see Figs 3b and 4d).

The independent local selection of one of the possible ambiguous matches cannot by itself explain why the ambiguous planes were suppressed in Fig. 2 and visible in Fig. 1. I will now briefly outline a computation scheme that is consistent with these results. Initially, surfaces are constructed that take into account all possible matches of all the pixels. Let the support

set of a surface be the set of points within a window of size W in one image, which contribute to its construction. Assume W is large enough so that random matches occur at all disparities. A surface is retained if it has a sufficiently large support set that is not included in the support set of a different surface. With simple transparencies (see refs 7 and 8) all possible solutions survive, as their support sets are disjoint. In the stereogram of the first experiment (Fig. 1) all four solutions survive because part of the support set of each solution is derived from random matches (as shown by the fact that the peaks in Fig. 4a are $\sim 10\%$ greater than the value of 0.5 expected from the strict matches shown in Fig. 1b). In the stereogram of experiment 2 (Fig. 2), the strong solution suppresses the others because its support set includes all the points in any region.

The main implication of the above discussion is that the resolution of matching ambiguities is not as simple as nearly all the computational theories of stereo vision assume. A unique solution is a stated objective of these theories. My results require their extension to describe surface interpolation, which allows and uses multiple matchings. Possibly, the processes involved in feature matching and the resolution of ambiguities are different from those involved in surface interpolation (see ref. 9). $\square$

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1. Yuille, A. L. & Poggio, T. A Generalized Ordering Constraint for Stereo Correspondence. Technical Report AIM-777 (Artificial Intelligence Laboratory, MIT, 1984).
2. Burt, P. & Julesz, B. *Perception* **11**, 621-624 (1982).
3. Kroll, J. D. & van de Grind, W. A. *Perception* **9**, 651-669 (1980).
4. Marr, D. & Poggio, T. *Proc. R. Soc. Lond.* **B204**, 301-328 (1979).
5. Mayhew, J. E. W. & Frisby, J. P. *Artif. Intell.* **17**, 349-386 (1981).
6. Akerstrom, R. A. & Todd, J. T. *Percept. Psychophys.* **44**, 421-432 (1988).
7. Prazdny, K. *Biol. Cybern.* **52**, 93-99 (1985).
8. Pollard, S. B., Mayhew, J. E. W. & Frisby, J. P. *Perception* **14**, 449-470 (1985).
9. Mitchison, G. J. *Vision Res.* **17**, 753-782 (1988).
10. Grimson, W. E. L. *From Images to Surfaces* (MIT Press, Cambridge, Massachusetts, 1981).
11. Nishihara, H. K. *Opt. Engng* **23**, 536-545 (1984).

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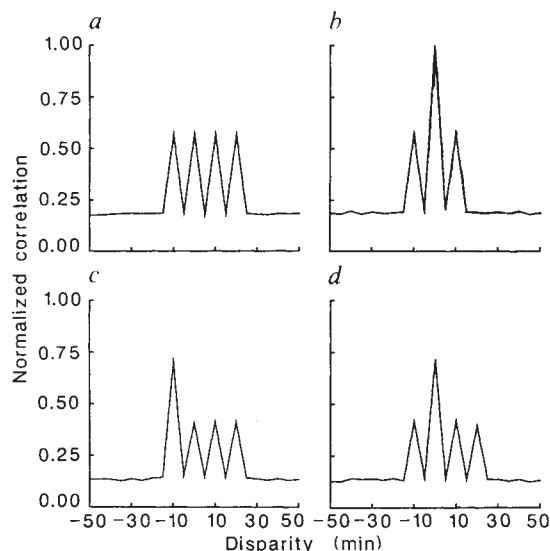


FIG. 4 The normalized correlation between the left and the right images at their centre: $\sum_i^W \sum_j^W I(x_i^r, y_j^r) I(x_i^l + D, y_j^l)$. The x-axis is the disparity D : a, Stereogram of Fig. 1; b, stereogram of Fig. 2; c, Stereogram of Fig. 3a; d, stereogram of Fig. 3b. The size of the window W is the width of the ambiguous square at the middle of each image. The correlation value is normalized by the number of features in the window so that it equals 1 if all the points are matched for some disparity value. These plots have been computed for stereogram with dot density of 10%. The noise level (10% in a) increases with dot density. Note that only in b, the only case where a single opaque plane is perceived, has one of the possible solutions a normalized disparity value of 1. This observation indicates a way to modify an area-based correlation stereo-matching algorithm to give the above results (for example, see ref. 11).

Arachidonic acid induces a long-term activity-dependent enhancement of synaptic transmission in the hippocampus

J. H. Williams, M. L. Errington, M. A. Lynch & T. V. P. Bliss

Division of Neurophysiology and Neuropharmacology, National Institute for Medical Research, Mill Hill, London NW7 1AA, UK

LONG-term potentiation (LTP) is a widely studied model of the synaptic basis of information storage in the mammalian brain^{1,2}. The induction of LTP is triggered by the postsynaptic entry of calcium through the channel associated with the *N*-methyl-D-aspartate (NMDA) receptor³⁻⁷, whereas its maintenance is mediated, at least in part, by presynaptic mechanisms⁸⁻¹². To explain how postsynaptic events can lead to an increase in transmitter release, we have postulated the existence of a retrograde messenger to carry information from the postsynaptic side of the synapse to recently active presynaptic terminals¹⁰. Candidates for a retrograde messenger include arachidonic acid or one of its lipoxygenase metabolites¹²⁻¹⁸. Here we report that weak activation of the perforant path, when given in the presence of arachidonic acid, leads to a slow-onset persistent increase in synaptic efficacy both *in vivo* and *in vitro*. The activity-dependent potentiation thus produced is accompanied by an increase in the release of glutamate, and is

non-additive with tetanus-induced LTP. These observations indicate a role for arachidonic acid as a retrograde messenger in the later, but not the initial, stages of LTP.

A push-pull cannula was used to perfuse the dentate gyrus in anaesthetized rats, while recording extracellular synaptic potentials evoked by test stimuli delivered to the perforant path. Addition of arachidonic acid (200 μ M) to the artificial cerebrospinal fluid for 35 min usually caused a slight and reversible depression of the population excitatory postsynaptic potential (e.p.s.p.), but did not produce long-term after-effects (Fig. 1a). In another group of animals, weak high-frequency trains, which given alone were subthreshold for the induction of LTP (Fig. 1b, first arrow), were repeated 5 min before the end of a 35 min period of perfusion with arachidonic acid (Fig. 1b, bar and second arrow). In all cases, the conjunction of weak trains and perfusion with arachidonic acid resulted in a slowly developing increase in the slope of the population e.p.s.p. The average potentiation ($\pm$ s.e.m.) 2 h after conjunction, calculated from the normalized mean values plotted in Fig. 1b, was $68.7 \pm 14.7\%$ (range 14.7–158.0%, $n = 7$). In another two animals, in which the initial weak train was omitted, the potentiation produced by conjunction persisted without decrease for 4 h (Fig. 1c). No potentiation occurred when the second train was given in the absence of arachidonic acid (data not shown).

To examine the possibility that a lipoxygenase metabolite of arachidonic acid, rather than arachidonic acid itself, is the active compound in producing potentiation, we applied a weak tetanus in the presence of arachidonic acid and the lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA, 200 μ M). The slow-onset potentiation was not affected and reached a magnitude, when measured 2 h after the weak tetanus, that was comparable to that seen in the tetanus-arachidonic acid group (Fig. 1d; $63.2 \pm 32.2\%$, range 14.9–81.2%, $n = 4$). An analogous result was obtained in the *in vitro* hippocampal slice preparation. These experiments indicate that it is arachidonic acid itself, rather than its lipoxygenase metabolites, that is important in generating the late potentiation. In four animals we examined the effect of AP5 (D(-)-2-amino-5-phosphonovaleate), an antagonist of the NMDA subtype of glutamate receptor and a potent blocker of the induction of LTP^{3,11}. When AP5 (100 μ M) was included in the perfusion medium during the period of perfusion with arachidonic acid, a similar slowly developing potentiation occurred after the weak tetanus (data not shown). We conclude that potentiation induced by arachidonic acid is not mediated by the NMDA receptor/channel.

The mean increase in the slope of the e.p.s.p. produced by the conjunction of weak stimulation and arachidonic acid is within the range of maximal LTP in this preparation¹⁹. The question arises whether the two effects exploit similar cellular mechanisms at some stage subsequent to the opening of the NMDA channel. We tested this possibility by occlusion experiments in four rats (Fig. 2). When strong tetani were given during the plateau phase of arachidonate-induced potentiation, there was no additional potentiation (Fig. 2a). In the converse experiment, maximal LTP was first induced by three episodes of strong tetanic stimulation. Arachidonic acid was then added to the perfusion medium and a series of weak tetani applied. There was no further increase in the slope of the e.p.s.p. (Fig. 2b). These observations imply a convergence in the sequence of events leading to potentiation in the two cases.

A similar persistent potentiation of synaptic efficacy was produced in the dentate gyrus of the hippocampal slice by the conjunction of arachidonic acid and weak activation. Because of the difficulty in establishing tetanus-induced LTP in the perforant path *in vitro*²⁰, we adopted an activation protocol in which the frequency of stimulation was increased from $\frac{1}{30}$ Hz to $\frac{1}{4}$ Hz for a 30 min period. With normal perfusion medium, stimulation at $\frac{1}{4}$ Hz caused an habituation²¹ of the response, which was only partially reversible (Fig. 3a). Addition of arachidonic acid (50 μ M) to the medium without increasing stimulus

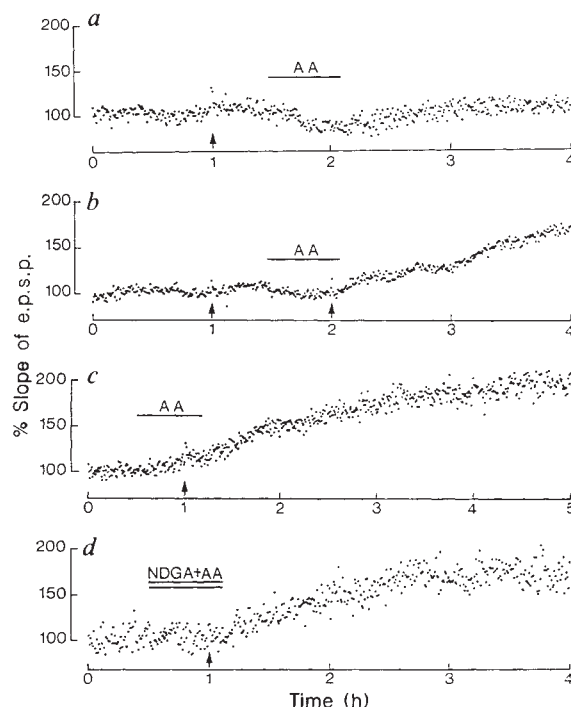


FIG. 1 Perfusion of arachidonic acid (AA) causes an activity-dependent potentiation of synaptic transmission in the dentate gyrus of the anaesthetized rat. *a–d*, Slope of the population e.p.s.p., recorded in the granule cell layer in response to stimulation with weak test shocks of the perforant path at $\frac{1}{30}$ Hz, is plotted against time. Values are normalized with respect to the mean e.p.s.p. slope for the 20 responses at the end of the first 60 min (*a, b*) or the first 30 min (*c, d*). The experimental design required the delivery of weak tetani that were subthreshold for the induction of LTP in normal medium; that this was the case was checked in the first part of the experiment (first arrows in *a, b*). *a*, Arachidonic acid alone (200 μ M) causes only a transient depression of the evoked response. Plot of the mean normalized e.p.s.p. in a group of three experiments in which no tetanic stimulation was given during the period of perfusion with AA (bar). *b*, Weak tetanus given in the presence of 200 μ M AA (bar) results in a slowly growing potentiation. The mean of seven experiments in which the first tetanus produced a potentiation of less than 10% is plotted. Note the slow onset and gradual climb towards a plateau in the 2-h period after the weak train. *c*, Mean of two experiments in which the potentiation produced by the conjunction of AA and a weak tetanus was followed for 4 h. *d*, Slow-onset potentiation produced by weak tetanic stimulation and AA (200 μ M) in the presence of NDGA (200 μ M). Mean of four experiments.

METHODS. Adult Sprague-Dawley rats were anaesthetized with urethane (1.5 g kg⁻¹, intraperitoneal) and placed in a stereotaxic headholder. A push-pull cannula (outer diameter 0.83 mm) with a pair of attached wire recording electrodes, was advanced into the dentate gyrus, so that the cannula straddled the molecular layer, with one recording electrode in the molecular layer and the other in the granule cell layer⁹. A bipolar stimulating electrode was advanced into the angular bundle to activate afferent fibres of the perforant path. Weak test shocks were alternated with stronger test shocks, above threshold for eliciting a population spike, so that changes in amplitude of the population spike could be monitored (not shown). Weak tetani consisted of three trains of stimuli (250 Hz for 200 ms, 60-s intertrain interval) at the same intensity as the weak test shocks. Responses were sampled digitally and stored on disk for later analysis. Artificial cerebrospinal fluid (ACSF, composition in mM: NaCl, 136; KCl, 2.54; KH₂PO₄, 1.2; CaCl₂, 1.8; MgSO₄·7H₂O, 1.18; NaHCO₃, 23; glucose, 10) was gassed with 95% O₂, 5% CO₂ and perfused throughout the experiment at 6.5 μ l min⁻¹. A period of 1 h elapsed between the beginning of perfusion and the collection of evoked potentials to allow the system to stabilize. AA (Sigma) was dissolved in ethanol by sonication. Aliquots, 1 mM in ACSF, containing ascorbic acid, were stored at -70 °C. For perfusion, aliquots were diluted in ACSF to a final concentration of AA, 200 μ M; ascorbic acid, 100 μ M, ethanol, 1%. Vehicle alone had no activity-dependent effects on evoked responses either *in vivo* or *in vitro*.

frequency resulted in an initial depression, followed by a transient potentiation on return to normal medium (Fig. 3b). The conjunction of arachidonic acid (50 μ M) and stimulation at $\frac{1}{30}$ Hz was followed by a rapid recovery from habituation and a gradual increase in the slope of the e.p.s.p. to well above control levels in the subsequent 1.5 h (Fig. 3c). Thus, as in the anaesthetized animal, it is the conjunction of increased activation and arachidonic acid that leads to potentiation. Despite the difference in stimulus protocols, the degree of potentiation was similar *in vitro* and *in vivo*.

We measured the release of glutamate into the perfusion medium in five of the *in vitro* experiments in which potentiation was produced by the conjunction of arachidonic acid and repetitive stimulation, and in five of the control experiments in which arachidonic acid was perfused without additional stimulation. (Similar measurements were not possible *in vivo*, because perfusion with arachidonic acid led to the appearance of blood in the perfusate, resulting in spuriously high glutamate concentrations). In the potentiated *in vitro* group, the mean glutamate concentration ($\pm$ s.e.m.) increased from 35.8 ± 3.8 nM in the first hour to 46.1 ± 2.5 nM in the third hour ($P < 0.05$, *t*-test); by contrast, the concentration of glutamate in the control group showed a non-significant decline from 40.6 ± 2.4 nM in the first hour to 35.9 ± 3.6 nM in the third hour. Interpretation of this result is complicated by the fact that the Schaffer-commissural pathway was activated with stimulus patterns identical to those applied to the perforant path (see Fig. 3 legend); a similar slow-onset potentiation was induced in this pathway after the conjunction of repetitive stimulation and arachidonic acid (results not shown). As a result, it is not possible to separate the contribution made by these two pathways to the total glutamate content in the perfusate. Nevertheless, we can conclude that in area CA1, or in the dentate gyrus, or more probably in both regions, potentiation induced by arachidonic acid is associated with an increase in glutamate release.

It has previously been shown that the two predominant *cis*-unsaturated fatty acids oleic acid and arachidonic acid, prolong a short-lasting form of LTP in the perforant paths¹⁶. The experiments reported here demonstrate that the conjunction of

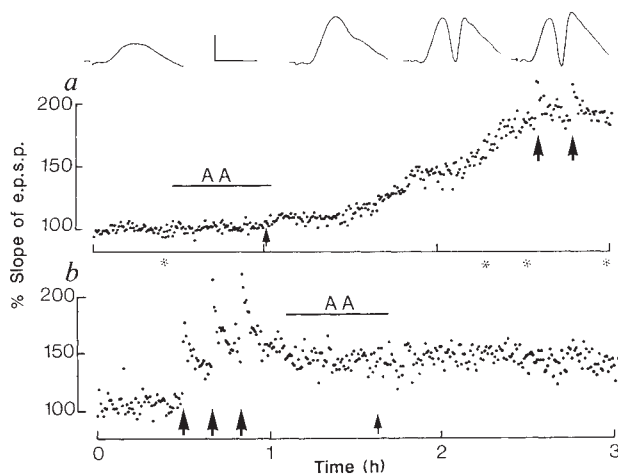


FIG. 2 LTP and activity-dependent arachidonate-induced potentiation are non-additive. *a*, Experiment in which no further potentiation was obtained when strong tetanic stimulation was given after AA-induced potentiation. The traces in the top panel were recorded at the times indicated by the asterisks under the abscissa. Each trace is the average of five consecutive responses to weak test shocks (calibration: 4 mV, 5 ms). Note the late development of a population spike. *b*, Experiment in which maximal LTP was induced by a series of three episodes of strong tetanic stimulation (250 Hz for 200 ms, intensity suprathreshold for population spike, 10 min between trains). Subsequent perfusion with AA and weak tetanic stimulation failed to induce further potentiation. Similar results were obtained in two other experiments.

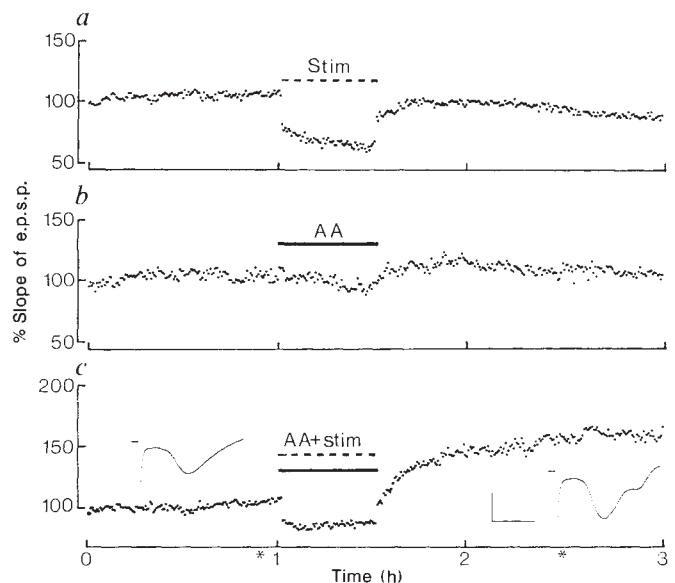


FIG. 3 Activity-dependent potentiation by arachidonic acid (AA) in the dentate gyrus of the hippocampal slice. *a-c*, slope of the population e.p.s.p. recorded in the molecular layer in response to test stimuli delivered to the perforant path at $\frac{1}{30}$ Hz is plotted against time. The intensity of test stimulation was subthreshold for evoking a population spike. Plots are group means, normalized with respect to the mean value of the e.p.s.p. in the 20 responses at the end of the first hour. *a*, Habituation produced by stimulation (stim) at $\frac{1}{30}$ Hz (stim, dashed line) in normal perfusion medium ($n=6$). *b*, AA alone (50 μ M) causes a transient slight depression of the population e.p.s.p. ($n=6$). *c*, AA (50 μ M) in conjunction with repetitive stimulation at $\frac{1}{30}$ Hz causes a persistent enhancement of synaptic transmission ($n=6$). The inset traces (means of five consecutive responses from one experiment) were obtained at the times indicated by asterisks under the abscissa (calibration: 1 mV, 5 ms).

METHODS. Transverse hippocampal slices (two per experiment) were maintained in a modified interface chamber at a temperature of 28–30 °C, and perfused with oxygenated ACSF at 100 μ l min⁻¹ as previously described¹³. In one of the two slices (the other being held as a reserve) stimulating electrodes were placed in the perforant path and in the Schaffer-commissural fibres, and recording electrodes in the molecular layer of the dentate gyrus and in stratum radiatum of area CA1. AA was prepared for perfusion as described in the legend to Fig. 1 to a final concentration of AA, 50 μ M; ascorbic acid, 25 μ M; ethanol, 0.25%. Evoked responses were digitized and stored on disk. Fractions of perfusate were collected every 15 min in a fraction collector packed with solid CO₂. Samples were stored at -70 °C, subsequently thawed, and derivatized with *o*-phthalaldehyde-mercaptopyropionic acid. Glutamate was separated from other amino acids by reverse-phase HPLC, and its concentration estimated fluorometrically with reference to a standard curve¹⁰.

exogenously applied arachidonic acid and weak afferent activation is sufficient to initiate a long-lasting increase in the efficacy of synaptic transmission in this pathway. As with tetanus-induced LTP, the enhancement of transmission is accompanied by, and is presumably at least partly due to, an increase in the release of glutamate. The occlusion experiments illustrated in Fig. 2 also indicate a convergence in the processes underlying arachidonate-induced and tetanus-induced LTP. Although our observations readily support the retrograde messenger hypothesis, according to which arachidonic acid is released from a postsynaptic site and acts on presynaptic terminals to increase transmitter release, we cannot exclude the possibility that arachidonate-induced potentiation is the result of an action on glial or postsynaptic sites. The time course of potentiation induced by arachidonic acid, with its slow onset and gradual climb towards a plateau during the 60–120 min after the washout of arachidonic acid, is in marked contrast to the rapid onset of LTP. Recent experiments with protein kinase inhibitors^{22–24} and inhibitors of protein synthesis^{25,26} reinforce earlier suggestions²⁷ that there are two or more temporal components of LTP—an early phase not blocked by protein synthesis or protein kinase

inhibitors, and a later phase blocked by both. Our results are consistent with a role for arachidonic acid as a retrograde messenger in the genesis of the later, but not the initial, component of LTP. The onset of increased transmitter release in LTP occurs not later than 15 min after the tetanus⁹⁻¹², so the present results imply either that the early phase of enhanced release is mediated by wholly presynaptic mechanisms, or that another messenger is involved. Possible candidates for a putative early messenger include endothelial-derived relaxing factor (EDRF, nitric oxide) which is rapidly released from cerebellar neurons by NMDA (ref. 28). □

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- Bliss, T. V. P. & Lomo, T. *J. Physiol., Lond.* **232**, 331-356 (1973).
- Landfield, P. W. & Deadwyler, S. A. (eds) *Long-term Potentiation: From Biophysics to Behavior* (Alan R. Liss, New York, 1988).
- Collingridge, G. L., Kehl, S. J. & McLennan, H. *J. Physiol., Lond.* **334**, 33-36 (1983).
- Gustafsson, B., Wigström, H., Abraham, W. C. & Huang, Y.-Y. *J. Neurosci.* **7**, 774-780 (1987).
- Kelso, S. R., Ganong, A. H. & Brown, T. H. *Proc. natn. Acad. Sci. U.S.A.* **83**, 5326-5330 (1986).
- Sastry, B. R., Goh, J. W. & Auyeung, A. *Science* **232**, 988-990 (1986).
- Kauer, J. A., Malenka, R. C. & Nicoll, R. A. *Nature* **334**, 250-252 (1988).
- Skrede, K. & Malthe-Sørensen, D. *Brain Res.* **208**, 436-441 (1981).
- Dolphin, A. C., Errington, M. L. & Bliss, T. V. P. *Nature* **297**, 496-498 (1982).
- Bliss, T. V. P., Douglas, R. M., Errington, M. L. & Lynch, M. A. *J. Physiol. Lond.* **377**, 391-408 (1986).
- Errington, M. L., Lynch, M. A. & Bliss, T. V. P. *Neuroscience* **20**, 279-284 (1987).
- Lynch, M. A., Errington, M. L. & Bliss, T. V. P. *Neuroscience* **30**, 693-701 (1989).
- Williams, J. H. & Bliss, T. V. P. *Neurosci. Lett.* **88**, 81-85 (1988).
- Piomelli, D. *et al. Nature* **328**, 38-43 (1987).
- Linden, D. H., Murakami, K. & Routtenberg, A. *Brain Res.* **379**, 358-363 (1986).
- Linden, D. J., Sheu, F.-S., Murakami, K. & Routtenberg, A. *J. Neurosci.* **7**, 3783-3792 (1987).
- Dumfries, A., Sebben, M., Haynes, L., Pin, J.-P. & Bockaert, J. *Nature* **336**, 68-70 (1988).
- Lazarewicz, J. W., Wroblewski, J. T., Palmer, M. E. & Costa, E. *Neuropharmacology* **27**, 765-769 (1988).
- Bliss, T. V. P., Errington, M. L. & Lynch, M. A. in *Excitatory Amino Acid Transmitters* (eds Hicks, T. P., Lodge, D. & McLennan, H.) 337-340 (Alan R. Liss, New York, 1987).
- Wigström, H. & Gustafsson, B. *Nature* **301**, 603-604 (1983).
- Teyler, T. J. & Alger, B. E. *Brain Res.* **115**, 413-426 (1976).
- Reymann, K. G., Brodermann, R., Kase, H. & Matthies, H. *Brain Res.* **461**, 388-392 (1988).
- Malinow, R., Madison, D. V. & Tsien, R. W. *Nature* **335**, 820-824 (1988).
- Malenka, R. C. *et al. Nature* **340**, 554-557 (1989).
- Frey, U., Krug, M., Reymann, K. G. & Matthies, H. *Brain Res.* **452**, 57-65 (1988).
- Otani, S., Marshall, C. J., Tate, W. P., Goddard, G. V. & Abraham, W. C. *Neuroscience* **28**, 519-526 (1989).
- Racine, R. J., Milgram, N. W. & Hafner, S. *Brain Res.* **260**, 217-231 (1983).
- Garthwaite, J., Charles, S. L. & Chess-Williams, R. *Nature* **336**, 385-388 (1988).

T cell depletion in transgenic mice carrying a mutant gene for TCR- β

Paul Krimpenfort^{*†}, Ferry Ossendorp[‡], Jannie Borst[‡], Cornelis Melief[‡] & Anton Berns^{*§}

^{*} Division of Molecular Genetics, The Netherlands Cancer Institute, and Department of Biochemistry, University of Amsterdam, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

[‡] Division of Immunology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

CLASSICAL T lymphocytes recognize foreign antigens in the context of self major histocompatibility complex (MHC) molecules by means of the T-cell receptor (TCR) $\alpha\beta$ heterodimer¹⁻³. The genes for TCR β -chains, like immunoglobulin genes, are subject to allelic exclusion. The introduction of a functional TCR- β gene into the germline of mice prevents rearrangement of endogenous TCR- β genes⁴. Here we report that the introduction of a non-functional TCR- β gene with a deletion of the major part of the variable region (ΔV -TCR- β), also inhibits endogenous TCR- β gene rearrangement. This inhibition is mediated via the encoded protein because impairment of endogenous TCR- β gene rearrangement is not found if a frameshift mutation is introduced into the DJ region of the ΔV -TCR- β transgene. The ΔV -TCR- β transgene can lead to two phenotypes, in which lymphoid development is perturbed. Phenotype A is characterized by a severe impairment

of both T and B cell development as reflected by the complete absence of certain lymphoid organs. In phenotype B, lymphoid organs are macroscopically normal, but T cell differentiation is impeded. Virtually all thymocytes lack membrane expression of TCR- $\alpha\beta$, but nevertheless carry the CD4 and CD8 antigens (CD4⁺CD8⁺ phenotype); they do not, however, mature further. The defect in mice of phenotype B but not of phenotype A can be corrected by the introduction of a functional TCR- β gene.

We investigated whether expression of a non-functional TCR- β gene inhibits endogenous TCR- β expression in transgenic mice. If so, the resulting mice would lack cells bearing functional TCR- $\alpha\beta$, which would be expected to influence the constellation of the immune system.

The relevant gene was isolated from a genomic library of the cytotoxic T cell clone B6.2.16 (ref. 4); this is specific for the male antigen HY and restricted by the H-2D^b molecule, and was previously used for the construction of transgenic mice^{4,5}. The cosmid clone cosHYB9-1.14-5 contains a functionally rearranged TCR- β gene, consisting of the V β 5.1 leader, V β 8.2, D β 2, J β 2.3 and the C β 2 gene segments. A 20-kilobase (kb) *KpnI* fragment derived from this clone was expressed at high levels in transgenic mice⁵. The non-functional gene was derived from this fragment by deletion of a 707-base pair (bp) *PstI* fragment, containing most of the V region and the complete VDJ junction. The reading frame and splice signals were restored by the insertion of two complementary synthetic oligonucleotides. The resulting 19.4-kb *KpnI* fragment (ΔV -TCR- β) encodes the V β 5.1 leader, a 12-amino-acid sequence from the N-terminal V β 8.2 region, the C-terminal J β 2.3 region and the C β 2 region (Fig. 1).

Four independent transgenic founders were obtained using the ΔV -TCR- β gene construct. The same construct gave rise to two different phenotypes, A and B (Table 1). Founders 1670, 1673 and 1733 exhibited phenotype A, whereas founder 1735 exhibited phenotype B. Offspring of founder 1673 were of either phenotype A or B. The A-phenotype offspring of founder 1673 had the same arrangement of the transgene as the founder, whereas B-phenotype offspring had lost a fraction of the transgenic copies. Founders 1733 and 1735 stably transmitted their transgenes.

Phenotype A is characterized by the complete absence of the thymus, lymph nodes and Peyer's patches in 8-week-old mice. In 5-month-old mice we occasionally observed a small thymus. Mice of phenotype A were highly sensitive to opportunistic infections. The transgene was highly expressed in the spleen of the A phenotype (Table 1). Immunofluorescence analysis demonstrated that in phenotype-A spleen, the percentage of T cells was greatly reduced from that of normal, as reflected by staining with the monoclonal antibodies anti-Thy1 (from 38% to 6%), anti-CD3 (from 38% to 13%), anti-CD4 (from 20% to 8%) and anti-CD8 (from 15% to 2.5%) (Fig. 2a). Splenic B cells defined by staining with anti-CD45R (B220) expressed normal levels of surface immunoglobulin, detected by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin antibodies (data not shown). In serum of A-phenotype mice, levels of IgM slightly lower than normal were observed, whereas IgG2a was not detectable and the levels of other IgG isotypes were less than normal. The cellular composition of bone marrow of A-phenotype mice was also abnormal. The percentage of cells expressing the CD45R (B220) marker was lower than in control mice; cells uniformly expressed the CD44 marker (Pgp1) and the percentage of CD11b (Mac1)-positive cells was higher than normal (Fig. 2b). No expression of the transgene was detected in the bone marrow (Table 1). The aberrant phenotype A could not be corrected by crossing-in functional TCR- β or $\alpha\beta$ transgenes.

Thus, in A-phenotype mice, development not only of T cells, but also of B cells is severely impaired, resulting in gross disorders such as the complete absence of certain lymphoid organs. It is not clear whether the impairment of B cell development is

[†] Present address: Genpharm BV, PO Box 9502, 2300 RA Leiden, The Netherlands.

[§] To whom correspondence should be addressed.

TABLE 1 TCR mRNA expression in phenotype A and phenotype B transgenic mice

mRNA	Phenotype A				Phenotype B			
	Spleen B-cells	T-cells	Thymus	Bone marrow	Spleen B-cells	T-cells	Thymus	Bone marrow
ΔV -TCR β	+	ND	NP	—	+	+	+	—
end β	—	ND	NP	—	—	+	<	—
end α	—	ND	NP	—	—	+	+	—

Abbreviations: end β , endogenous 1.3-kb TCR- β mRNA; end α , endogenous TCR- α mRNA; ND, not determined; <, strongly reduced.

due to T cell deficiency (for example, lack of helper T cell subsets producing B cell growth factors or differentiation factors, or both) or if it is due to a direct effect of the expression of the transgene in B cells. The A phenotype was not observed in more than 25 independent transgenic lines that expressed a functional TCR- β gene; this phenotype must, therefore, be a result of specific features of the ΔV -TCR- β chain.

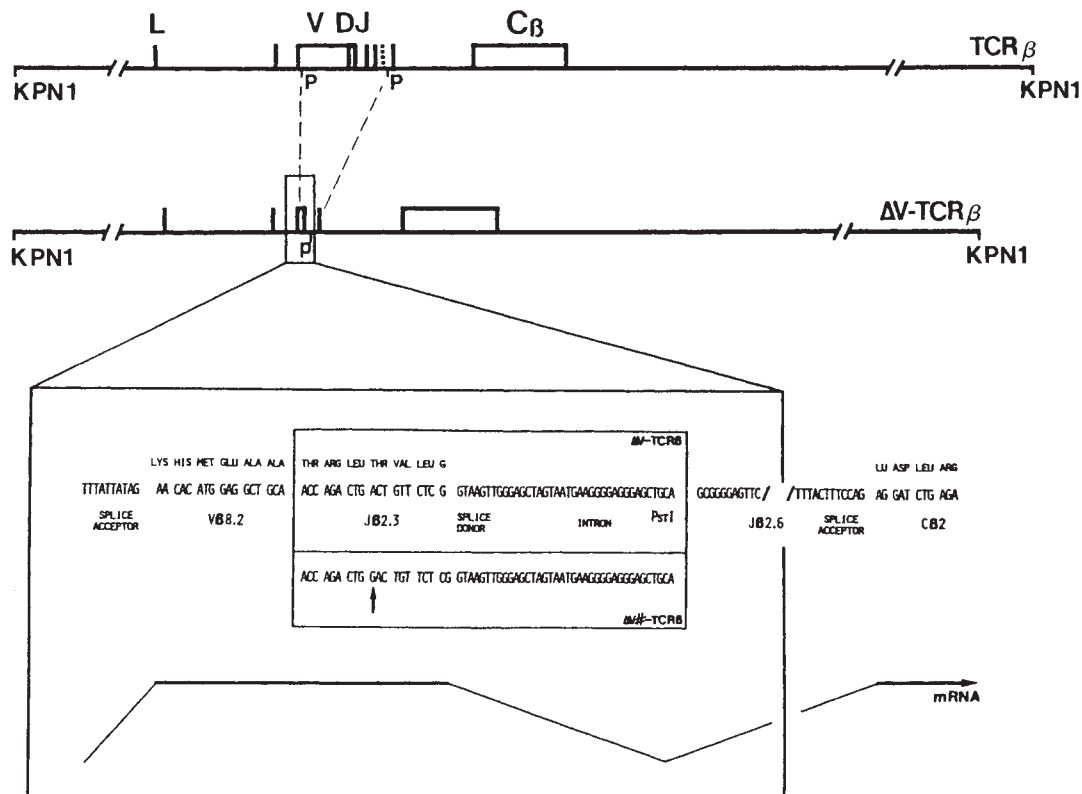
In B-phenotype mice, all lymphoid organs were macroscopically normal. In the thymus, the ΔV -TCR- β transgene was expressed at high levels, whereas the expression of the 1.3-kb mature messenger RNA for the endogenous TCR- β , but not the mRNA for TCR- α , was strongly suppressed (data not shown). In this respect thymocytes of B-phenotype mice resemble pre-B cells, in which heavy- but not light-chain expression is inhibited by the $D_{\mu}J_{\mu}C_{\mu}$ protein product of a partially rearranged heavy-chain gene⁶. In mice that carry a functional TCR- β transgene, suppression of endogenous TCR- β gene expression in the thymus appeared to be due to the incomplete rearrangement of the VDJ regions⁴. When DNA from thymocytes of B-phenotype

mice was analysed by Southern blotting using a D β 1 probe, a series of bands representing incomplete TCR- β gene rearrangements was detected (Fig. 3a). This suggests that introduction of the ΔV -TCR- β gene construct impairs TCR- β gene rearrangement according to a mechanism similar or identical to that involved when a TCR- β gene construct encoding a functional TCR- β chain is introduced.

The number of thymocytes in mice of phenotype B was normal. Thymocytes from B-phenotype mice and normal controls were stained with a panel of T cell-specific monoclonal antibodies (Fig. 3b). Whereas in normal mice ~15% of thymocytes expressed CD3 antigen at high levels (CD3-bright cells), in thymuses of B-phenotype mice only a small percentage (2–3%) of CD3-bright cells were detected. Similarly, whereas 85% of normal thymocytes expressed CD3 antigen at low levels (CD3-dull cells) or not at all (CD3-negative cells), the CD3-dull population was virtually absent in thymocytes from B-phenotype mice. The same observation was made for expression of TCR- $\alpha\beta$ (data not shown). Protein chemical analysis of thymocyte popu-

FIG. 1 Construction of the mutant TCR β -chain genes. The upper line illustrates the 20-kb *Kpn*I fragment derived from the cosmid clone cosHY β 9.114-5 containing the functionally rearranged TCR- β gene expressed in the B6.2.16 clone. The second line shows the structure of the mutant TCR- β genes, present on 19.4 kb *Kpn*I fragments used for injection. The region of interest is enlarged below to show in detail the region mutated in the ΔV -TCR- β constructs; the sequences inserted by the oligomers in the ΔV constructs are boxed and the additional nucleotide in the $\Delta V\#$ -TCR- β construct is indicated by an arrow.

METHODS. The 20 kb *Kpn*I fragment from cosHY β 9.1.14-5 was subcloned in pUC18; the functional TCR- β gene from B6.2.16 contains, in addition to the fused V β 8.2, D β 2.3 and J β 2 segments, the J segments J β 2.4, J β 2.5, ψ J β 2 and J β 2.6; a 4.0-kb *Xba*I fragment containing the VDJ join and the remaining J segments was inserted into pSP64; in this fragment three *Pst*I sites are present, the first at 17 bp from the start of the V β 8.2 segment, the second between the J β 2.5 and the ψ J β 2 segments and the third 22 bp after the ψ J β 2 segment; the 707 bp between the first and third *Pst*I site were deleted from the *Xba*I fragment; two complementary oligonucleotides were synthesized (Millipore Cyclone) corresponding to the last six codons of the J β 2.3 segment and the 34 bp downstream region from this segment, including the splice donor sequences. These were inserted in the *Pst*I site and replaced the 707-bp *Pst*I fragment; the sequence was chosen such that annealed oligomers carried a *Pst*I overhang at the borders, but correct insertion only restored the 3' *Pst*I site; by means of several subcloning steps, the annealed



oligomers were introduced into the *Xba*I fragment, which was inserted into the large *Kpn*I fragment present in pUC18; all manipulations involving the mutation of the VDJ region were checked by nucleotide sequence analysis. For injection, the DNA inserts were released from the vector by *Kpn*I digestion and purified as described⁵ and transgenic (CBA/BR $\times$ C57BL/LiA) F₂ mice were generated by the pronuclear injection technique; transgenic founders were backcrossed with C57BL/LiA (H-2^b) mice; screening by Southern blotting of tail DNA using a J β 2-specific probe as described⁵; the J β 2-specific probe recognizes a 2.6-kp *Eco*RV fragment in germline DNA, whereas an 1.1 kb *Eco*RV fragment is diagnostic for the ΔV constructs.

lations depleted of CD3-bright cells confirmed the absence of a TCR-CD3 complex at the cell surface (data not shown). Despite the lack of TCR- $\alpha\beta$, the thymocytes uniformly expressed both CD4 and CD8 antigens (Fig. 3b). The very small population of CD3-bright cells appeared to comprise CD4⁺CD8⁻, CD4⁻CD8⁺ as well as CD4⁻CD8⁻ cells.

In the spleen of 8-week-old B-phenotype mice, the percentage of T cells was reduced by about twofold compared with control mice, as reflected by staining with the monoclonal antibodies anti-CD3 (from 41% to 23%), anti-CD4 (from 24% to 11%) and anti-CD8 (from 15% to 8%). By 6 months of age, the percentage of T cells had reached normal levels. Purified splenic T cells stained normally with anti-CD3, anti-CD4 and anti-CD8 antibodies, as well as with antibodies specific for the TCR-V β 8 region (F23.1)⁷ and a common determinant on TCR- $\alpha\beta$ ¹¹ (data not shown). In purified T cells from the spleen, transcription of the transgene and also of endogenous TCR- β and α genes occurred (Table 1). Apparently, in these T cells, expression of the transgene has not inhibited endogenous TCR- β gene rearrangement. The marker profile of B-phenotype bone marrow was normal (not shown).

To investigate whether the mRNA of Δ V-TCR- β or the polypeptide encoded by Δ V-TCR- β caused inhibition of endogenous TCR- β gene rearrangement, resulting in the observed reduction of mature CD3 antigen-expressing cells in mice of both phenotypes, a Δ V β -TCR- β clone was constructed that carried an additional nucleotide in the region of the synthetic oligomers (Fig. 1). In this clone the C β segment is no longer in frame with the AUG initiation codon. Two transgenic founders were obtained with this construct. Thymus, spleen and lymph nodes were normal (macroscopically, with respect to numbers of B and T cells, and as judged by immunofluorescence analysis with specific antibodies). The Δ V β -TCR- β transgene, but also endogenous TCR- β genes were transcribed at high levels in these mice (data not shown), indicating that the mRNA of Δ V β -TCR- β by itself is stable but unable to inhibit endogenous TCR- β gene rearrangement. This strongly suggests that inhibition of the endogenous TCR- β gene rearrangement is mediated by the protein encoded by the Δ V-TCR- β transgene.

We conclude that in B-phenotype mice, differentiation of TCR- $\alpha\beta$ cells is impaired owing to the absence of TCR- $\alpha\beta$

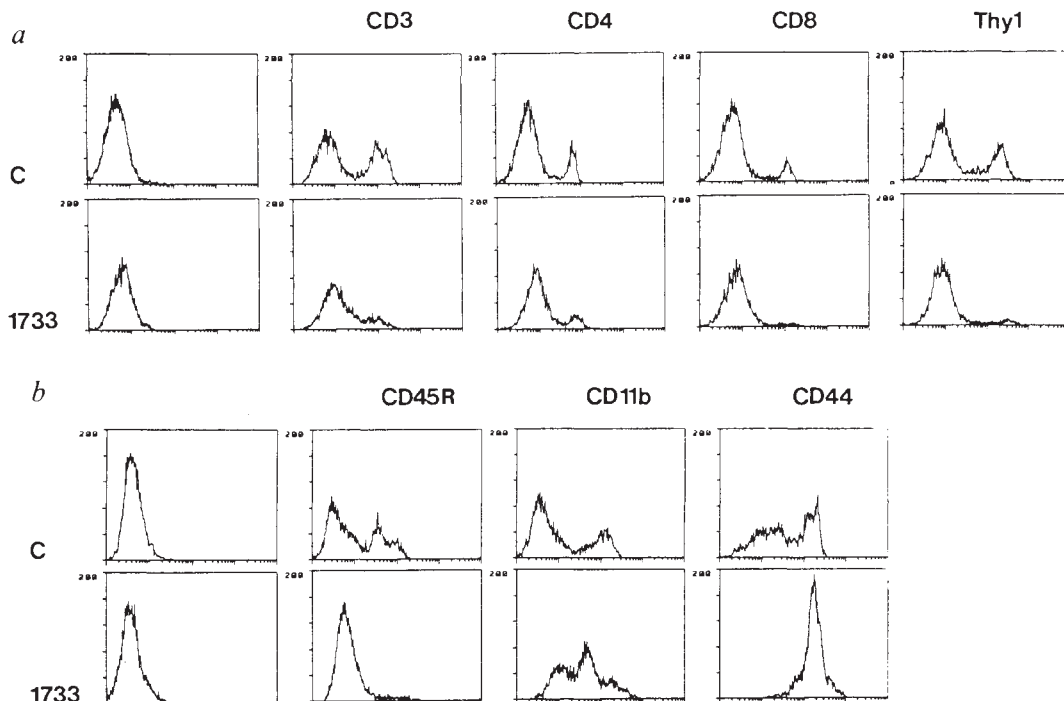
protein on the plasma membrane of thymocytes. In the vast majority of B-phenotype thymocytes, endogenous TCR- β protein is not synthesized, because the transgene inhibits endogenous TCR- β gene rearrangements. Consequently, thymocytes do not mature to the single positive stage because of the lack of positive selection in the absence of a functional TCR $\alpha\beta$ at the cell surface⁸⁻¹⁰. Because the generation of mature T cells is significantly impeded, spleens of B-phenotype young mice contain fewer T cells than those of normal young mice. The T cells that do reach the spleen have escaped inhibition of TCR- β rearrangement and do express a functional TCR- $\alpha\beta$. These cells probably originate from the few CD3-bright cells seen in the thymus. It has not yet been investigated whether the TCR $\gamma\delta$ ⁺ population is affected in B-phenotype mice, but clearly no major expansion of this subset has occurred. This would have resulted in a discrepancy in reactivity with anti-CD3 and anti-TCR $\alpha\beta$ antibodies.

To confirm that the impairment of thymocyte maturation in B-phenotype mice was indeed caused by the absence of a functional TCR β -chain, these mice were crossed with transgenic mice carrying a functional TCR- β gene (strain 101, expressing the cosHY β 9-1.14-5 construct⁵). Figure 3d shows the immunofluorescence data obtained with thymocytes from the double transgenic offspring. Clearly, the CD3-bright thymocyte population has been restored, and it also expresses TCR $\alpha\beta$ heterodimer. This receptor is composed of the products of the functional TCR- β transgene and endogenous TCR α -chains as demonstrated by its reactivity with V β 8-specific antibody F23.1⁷. Expression patterns of antigens CD4 and CD8 were indistinguishable from those of the 101 line. Apparently, expression of TCR $\alpha\beta$ -CD3 at the plasma membrane restores differentiation of thymocytes, yielding mice of the CD4⁺CD8⁻ and CD4⁻CD8⁺ mature thymocyte subsets. Spleens of these mice contained normal numbers of T cells, all of which stained with F23.1 antibody (Fig. 3e) and expressed both transgenes (data not shown).

By using TCR $\alpha\beta$ -transgenic severe combined immunodeficiency (SCID) mice, Scott *et al.*¹² have recently shown that the transition of CD4⁻CD8⁻ thymocytes to double positive CD4⁺CD8⁺ thymocytes requires the productive rearrangement of TCR gene segments. Here we have shown that cell-surface

FIG. 2. FACS analysis of splenocytes (a) and bone-marrow cells (b) from 8-week-old non-transgenic control (C) and phenotype-A mice (strain 1733). Horizontal axis, staining intensity; Vertical axis, number of cells. Antibodies used: FITC-conjugated mouse anti-rat kappa-chain mAb (-); 145-2C11¹³ (anti-CD3); H129-19¹⁴ (anti-CD4); 53-6.7¹⁵ (anti-CD8); 59AD2.2 (anti-Thy1); RA3-6B2¹⁶ (anti-CD45R); M1/70.15¹⁷ (anti-CD11b); and Pgp1¹⁸ (anti-CD44).

METHODS. Single-cell suspensions were prepared from spleen (a) or bone marrow (b); erythrocytes were lysed, washed in PBS containing 0.5% w/v BSA and 0.05% sodium azide, counted, and incubated with saturating amounts of antibodies and then stained with FITC-conjugated mouse anti-rat kappa-chain mAb or stained directly with FITC-conjugated 145-2C11 (anti-CD3) mAb; cells were washed three times and analysed using a FACScan (Becton Dickinson) flow cytometer; 5,000 viable cells were analysed in each sample.



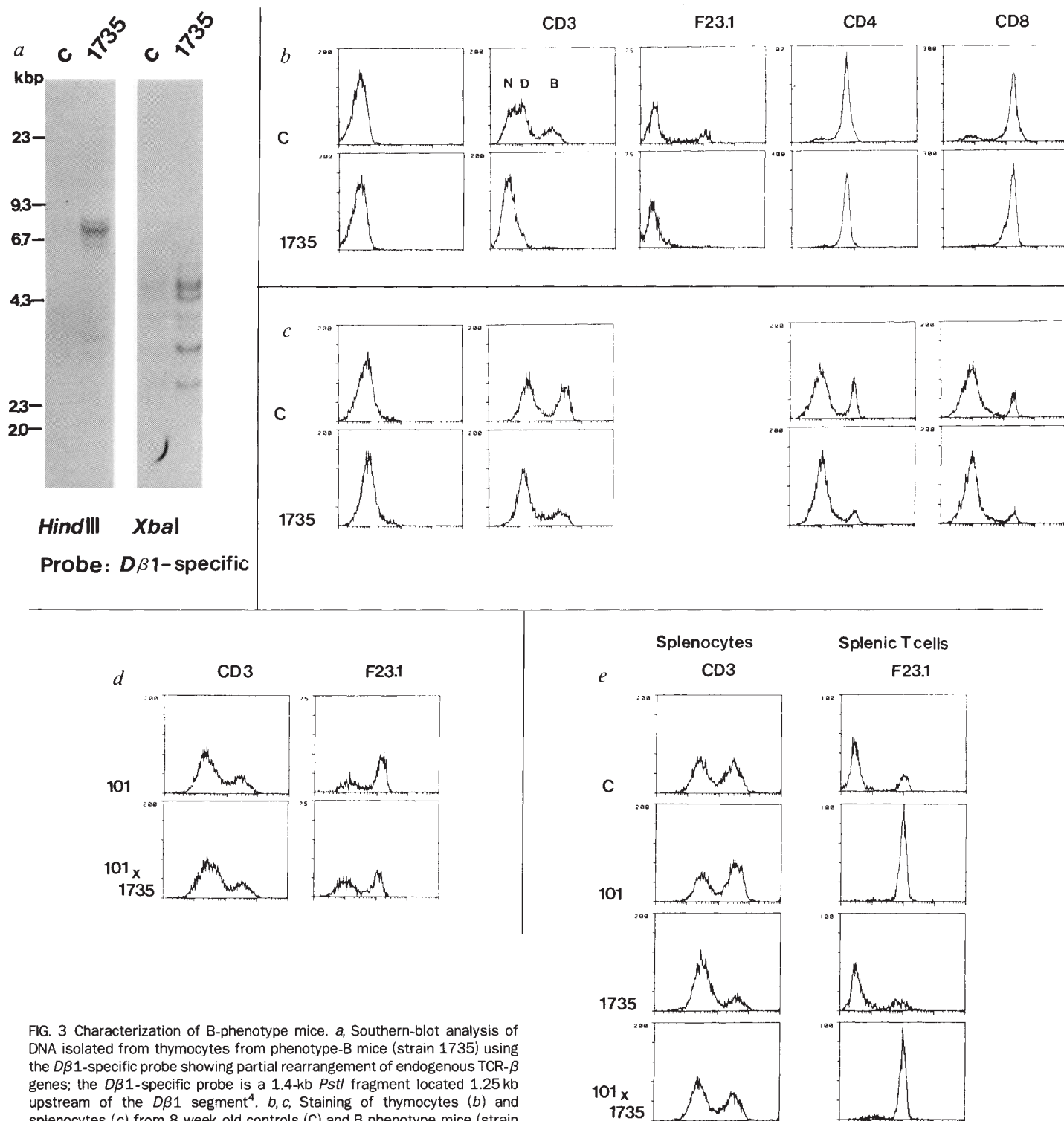


FIG. 3 Characterization of B-phenotype mice. **a**, Southern-blot analysis of DNA isolated from thymocytes from phenotype-B mice (strain 1735) using the $D\beta 1$ -specific probe showing partial rearrangement of endogenous TCR- β genes; the $D\beta 1$ -specific probe is a 1.4-kb *PstI* fragment located 1.25 kb upstream of the $D\beta 1$ segment⁴. **b, c**, Staining of thymocytes (**b**) and splenocytes (**c**) from 8-week-old controls (C) and B-phenotype mice (strain 1735). Antibodies used: 145-2C11 (anti-CD3), F23.1 (anti-V $\beta 8$), H129.19 (anti-CD4) and 53-6.7 (anti-CD8). N, CD3 negative; D, CD3-dull; B, CD3 bright. **d, e**, Restoration of T-cell maturation in ΔV -TCR- β mice by functional TCR- β transgene. Staining of thymocytes (**d**) and total splenocytes and purified T cells from spleen (**e**) from 8-week-old mice with anti-CD3 and F23.1 antibodies. Mice of strain 101 (carrying the cosHY $\beta 9$ -1.14-5 transgene) and strain 1735 were crossed to generate transgenic mice carrying either only the complete functional TCR- β transgene (101) or both transgenes (1735 $\times$ 101).

METHODS. For Southern-blot analysis, 8 μ g total genomic DNA was digested with restriction endonucleases, separated on agarose gels and transferred to nitrocellulose; filters were hybridized to ³²P-labelled probes as described⁵. Mice were screened by tail-DNA analysis using the $J\beta 2$ -specific probe; this probe recognizes a 2.6-kb fragment in germ-line DNA, whereas a 2.3-kb

fragment and a 11-kb fragment are diagnostic for the functional TCR- β transgene and the ΔV -TCR- β transgene, respectively; purified T cells were isolated by depletion of B cells; splenocytes were incubated with magnetic beads coated with goat anti-mouse immunoglobulin antibody (DynaI, Norway) for 1 h at 4 °C and the rosetted B cells were removed with a magnet. Preparation of cell suspensions and FACS analyses were performed as described in the legend to Fig. 2; cells were incubated with H129.19 (anti-CD4) and 53.6.7 (anti-CD8) mAbs and stained with FITC-conjugated mouse anti-rat kappa-chain mAb, or with F23.1 and stained with FITC-conjugated goat anti-mouse immunoglobulin (Tago), or stained directly with FITC-conjugated 145-2C11 (anti-CD3) antibody.

expression of functionally rearranged TCR- $\alpha\beta$ is not required for the induction of the expression of CD4 and CD8 antigens. It remains to be shown whether the intracellular expression of defective TCR- β and endogenous TCR- α is required for T cell development to advance to the double positive stage. $\square$

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1. Marrack, J. & Kappler, P. J. *Adv. Immun.* **38**, 1-30 (1986).
2. Kronenberg, M., Siu, G., Hood, L.E. & Shastri, N. A. *Rev. Immun.* **4**, 529-591 (1986).
3. Davis, M. M. & Bjorkman, P. J. *Nature* **334**, 395-402 (1988).
4. Uematsu, Y. et al. *Cell* **52**, 831-841 (1988).
5. Krimpenfort, P. et al. *EMBO J.* **7**, 745-750 (1988).
6. Reth, M. G., Ammirati, P., Jackson, S. & Alt, F. W. *Nature* **317**, 353-355 (1985).
7. Staerz, U. D., Rammensee, H. G., Benedetto, J. D. & Bevan, M. J. *J. Immun.* **134**, 3994-4000 (1985).
8. Kisielow, P., Blüthmann, H., Staerz, U. D., Steinmetz, M., & Von Boehmer, H. *Nature* **333**, 742-746 (1988).
9. Teh, H. S. et al. *Nature* **335**, 229-233 (1988).
10. Sha, W. C. et al. *Nature* **336**, 73-76 (1988).
11. Kubo, R. T. et al. *J. Immun.* **142**, 2736-2742 (1989).
12. Scott, B., Blüthmann, H., Teh, H. S. & Von Boehmer, H. *Nature* **338**, 591-593 (1989).
13. Leo, O. et al. *J. Immun.* **84**, 1374-1378 (1987).
14. Pierres, A. et al. *J. Immun.* **132**, 2775-2780 (1984).
15. Ledbetter, J. A. *Immunol. Rev.* **47**, 67-78 (1979).
16. Coffman, R. L. *Immunol. Rev.* **69**, 5-23 (1982).
17. Trowbridge, L. S. & Omary, M. J. *exp. Med.* **154**, 1517-1524 (1981).
18. Picker, L. et al. *J. Immun.* **142**, 2046-2051 (1989).

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Selective development of CD4⁺ T cells in transgenic mice expressing a class II MHC-restricted antigen receptor

Jonathan Kaye, Mei-Ling Hsu, Marie-Elizabeth Sauron, Stephen C. Jameson*, Nicholas R. J. Gascoigne* & Stephen M. Hedrick

Department of Biology and Cancer Center, University of California, San Diego, La Jolla, California 92093, USA.

* Department of Immunology, Research Institute of Scripps Clinic, La Jolla, California 92037, USA

T LYMPHOCYTES are predisposed to recognition of foreign protein fragments bound to cell-surface molecules encoded by the major histocompatibility complex (MHC)^{1,2}. There is now compelling evidence that this specificity is a consequence of a selection process operating on developing T lymphocytes in the thymus³⁻⁶. As a result of this positive selection, thymocytes that express antigen receptors with a threshold affinity for self MHC-encoded glycoproteins preferentially emigrate from the thymus and seed peripheral lymphoid organs. The specificity for both foreign antigen and MHC molecules is imparted by the α and β chains of the T-cell antigen receptor (TCR)^{7,8}. Two other T-cell surface proteins, CD4 and CD8, which bind non-polymorphic regions of class II and class I MHC molecules respectively^{9,10}, are also involved in these recognition events and play an integral role in thymic selection. In order to elucidate the developmental pathways of class II MHC-restricted T cells in relation to these essential accessory molecules, we have produced TCR-transgenic mice expressing a receptor specific for a fragment of pigeon cytochrome c and the E^k (class II MHC) molecule. The transgenic TCR is expressed on virtually all T cells in mice expressing E^k. The thymuses of these mice contain an abnormally high percentage of mature CD4⁺CD8⁻ cells. In addition, the peripheral T-cell population is almost exclusively CD4⁺, demonstrating that the MHC specificity of the TCR determines the phenotype of T cells during selection in the thymus.

A large number of cell surface proteins have now been identified which function in the cellular interactions of T lymphocytes. Central among these is the antigen receptor, which enables T cells to detect foreign peptides bound to MHC proteins on the surface of other cells. The major population of T lymphocytes express antigen receptors comprised of disulphide bonded α and β chains noncovalently associated with four or five other polypeptide chains of the CD3 complex (reviewed in ref. 11). T cells bearing $\alpha\beta$ TCR can be divided into two subsets, based on their specificity for class I or class II MHC proteins. The CD4 and CD8 molecules, expressed on cells specific for class II or class I MHC molecules respectively, also delineate these T-cell subsets¹². These MHC-binding molecules are thought to associate with the $\alpha\beta$ TCR, enhancing the avidity of cell to cell interactions as well as playing a role in T-cell activation¹³⁻¹⁵. Peripheral T cells arise from thymic CD4⁺CD8⁺ precursors, so called double positive cells^{16,17}. As a given T cell undergoes thymic maturation, one of these cell surface proteins is downregulated resulting in a CD4⁺CD8⁻ or CD4⁻CD8⁺ phenotype. By an unknown mechanism the differentiation of thymocytes to these phenotypes is intimately linked to the positive selection of T cells expressing class I or class II MHC specific receptors. One approach to understanding these developmental mechanisms is the use of TCR transgenic mice which possess an extremely limited TCR repertoire. Recent reports which described transgenic mice expressing $\alpha\beta$ receptors specific for class I MHC gene products^{18,19} have shown that peripheral T cells bearing the transgene-encoded receptor were highly skewed toward expression of CD8. In this report, we examine the development of the other major subpopulation of T cells, those restricted to class II MHC proteins, utilizing mice transgenic for an E^k restricted TCR.

(C57B16/J $\times$ SJL/J)_F₂ transgenic mice were produced by microinjection of the α and β TCR gene constructs shown in Fig. 1. Both transgenes contained endogenous promoter and enhancer sequences, although the position of the β -chain enhancer was altered as was the original V $_{\alpha}$ -C $_{\alpha}$ distance. The productively rearranged α chain gene (V $_{\alpha}$ 11.1-J $_{\alpha}$ 84) was derived from the T-cell clone AN6.2 (ref. 20) while the β chain gene (V $_{\beta}$ 3-J $_{\beta}$ 1.2) was derived from T cell clone 5C.C7 (ref. 21). Both these T-cell lines are specific for the carboxy-terminal fragment of pigeon cytochrome c and the E^k molecule, although they exhibit

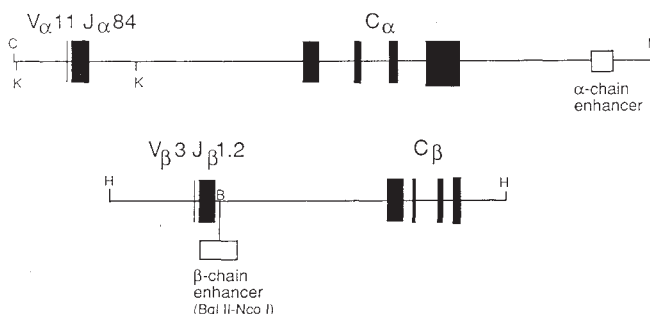


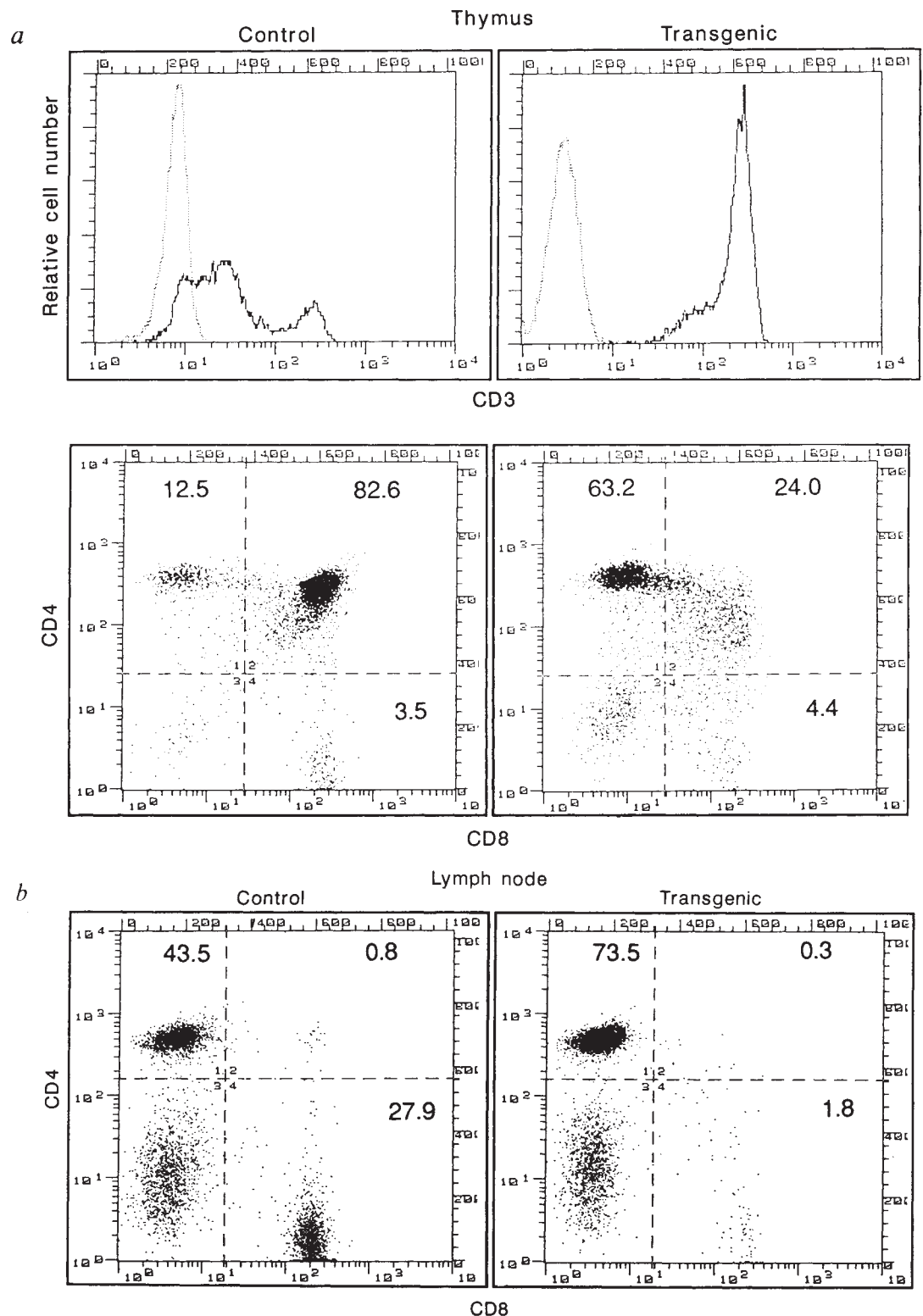
FIG. 1 TCR transgenes. A genomic clone containing a productively rearranged V $_{\alpha}$ -J $_{\alpha}$ gene derived from T-cell clone AN6.2 (ref. 20) was a generous gift from A. Winoto (Whitehead Institute). A 6.6-kilobase (kb) *Sal* I fragment containing the V $_{\alpha}$ -J $_{\alpha}$ was subcloned into the plasmid vector pBS (Stratagene) and a 3.3 kb fragment was isolated utilizing a *Kpn* I site 1.2 kb 5' to the V and a 3' *Kpn* I site in the multiple cloning site. This fragment was cloned into the *Kpn* I site of a pBS-based plasmid (modified with a 5' *Clal* site and a 3' *Not* I site) containing the complete genomic C $_{\alpha}$ gene²⁵ including the recently identified enhancer sequence²⁶, kindly provided by M. Davis (Stanford University). The rearranged genomic β chain gene derived from T-cell clone 5C.C7 (ref. 21) was isolated as previously described²⁷, and a 913 base pair *Bgl* II-*Nco* I fragment containing the β chain enhancer²⁸ was cloned into the J-C intron at the *Bam* HI site with a *Bgl* II linker. The 15.2 kb *Cla* I-*Not* I fragment containing the complete α -chain gene and the 9.8 kb *Hind* III fragment containing the complete β -chain gene were microinjected in equimolar amounts by standard procedures²⁹.

fine specificity differences between them. This particular α and β TCR pair has been expressed *in vitro* by gene transfer and the resulting transfectants maintain specificity for pigeon cytochrome c and E^k (J.K., unpublished data). In fact, this TCR differs from one found in a naturally occurring pigeon cytochrome c specific T cell by only a single amino acid in the V-J junction of the α -chain (clone AD10 in ref. 22).

Of 56 mice produced, 14 were found to have incorporated both α and β transgenes into their genome. Results from the progeny of one male founder mouse possessing approximately 2 copies of the α transgene and 1 copy of the β transgene are

presented here. This founder mouse was mated to a B10.A mouse and the offspring were H-2 typed using anti-H-2K monoclonal antibodies. The results from (H-2^b × H-2^a)F₁ progeny which express A^k, A^b, E^k and E^b class II MHC proteins are discussed below. Cell yields of thymocytes, lymph node cells and spleen cells were similar from transgenic and normal mice. The thymocytes from a 6 week-old transgenic mouse and a normal littermate were stained for CD3, or CD4 and CD8 and analysed by flow cytometry (Fig. 2a). In the normal mouse three populations of TCR bearing cells can be distinguished, as determined by expression of CD3. Approximately 25% of total thymocytes

FIG. 2 Phenotype of T cells in normal and $\alpha\beta$ transgenic mice. *a*, (Upper panels) thymocytes from a 6-week-old $\alpha\beta$ transgenic mouse or a normal littermate were stained with the anti-CD3 monoclonal antibody 2C11 (ref. 30) followed by FITC-conjugated goat anti-hamster Ig (Caltag) (solid line). Control staining was with secondary antibody alone (broken line). Cells were analysed on a FAC-Scan flow cytometer (Becton Dickinson) using FACScan Research Software. Dead cells were eliminated from the analysis on the basis of forward and sideways light scatter. Each plot represents the results from 5,000 viable cells. (Lower panels), thymocytes were stained with a mixture of PE conjugated anti-CD4 and FITC conjugated anti-CD8 (Becton Dickinson). Cells were analysed as above. The percentage of total cells in each quadrant is indicated. *b*, Lymph node cells were stained with anti-CD4 and anti-CD8 as described above.



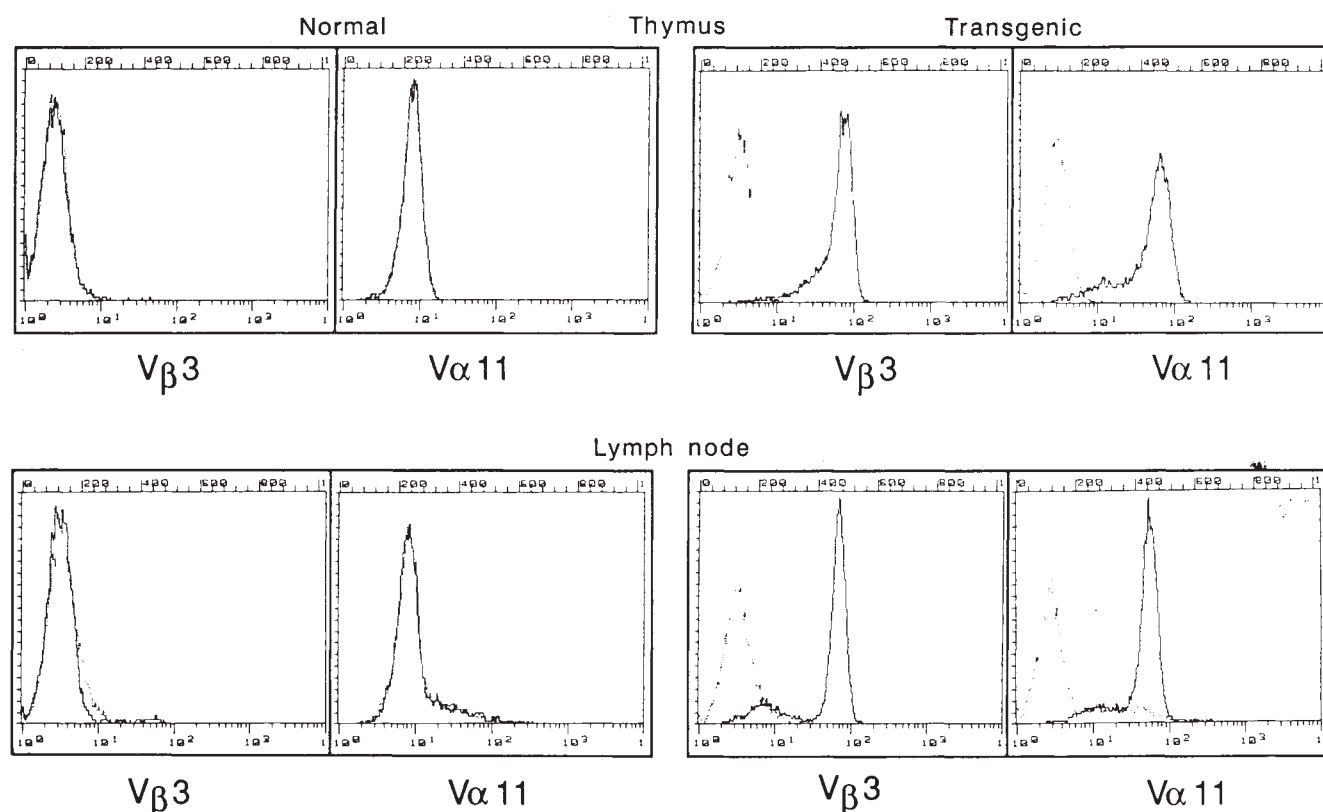


FIG. 3 Expression of $\alpha\beta$ transgenes in thymus and lymph node. Thymocytes or lymph node cells from a transgenic or normal littermate mouse were stained with FITC-conjugated anti- $V\beta 3$ monoclonal antibody KJ25 (ref. 24) (solid line) or FITC-conjugated normal rat Ig (broken line) as a control.

Alternatively, cells were stained with the hamster anti- $V\alpha 11$ monoclonal antibody 1F2 followed by FITC-conjugated goat anti-hamster Ig (Caltag) (solid line). Controls for these stainings were with secondary reagent alone (broken lines). Cells were analysed as described in Fig. 2.

express no detectable CD3, 57% express low levels and a small population (18%) expresses high levels of CD3 (Fig. 2a). This progression of TCR expression from low to high is a component of normal thymocyte maturation²³. In sharp contrast, thymocytes from transgenic mice (Fig. 2a) are 99% CD3⁺, 75% of these staining brightly and 25% somewhat duller. Unlike normal thymocytes, therefore, the TCR in these transgenic mice is expressed throughout thymocyte development, although there is a modulation of expression level as in normal mice. Similar observations have been reported for other $\alpha\beta$ transgenic mice¹⁸. The fact that TCR transgenes do not have to undergo the process of V-J rearrangement as do endogenous TCR genes may result in their accelerated expression.

The CD4/CD8 phenotypes are also altered in the transgenic mice, which express a higher than normal percentage of CD4⁺CD8⁻ thymocytes (63% compared with 13%) and a lower than normal percentage of double positive cells (24% compared with 83%) (Fig. 2a). Although the CD4⁺CD8⁺ populations in normal and transgenic mice appear similar as presented in Fig. 2a, a more stringent gating to eliminate potential CD4^{low}CD8⁺ cells suggests that there is also a twofold reduction in CD8 single positive cells. An increase in double negative cells is also found in the transgenic thymus (Fig. 2a), although the significance of this observation is unclear. Both the CD3 profile and the CD4/CD8 phenotyping are consistent with the presence of a larger than normal fraction of mature thymocytes in these $\alpha\beta$ transgenic mice. In addition, the mature thymocytes are greatly skewed towards expression of CD4. The steady state decrease in the size of the double positive population and the corresponding increase in CD4 single positive cells is consistent with the presence of a positive selection process that is more efficient than normal. The fact that the vast majority of T cells in this mouse expresses the transgenic TCR (see below) supports this idea. Unlike a normal mouse, where positive and negative

selection of cells bearing random TCR's presumably eliminate many potential T cells, the selection of these transgenic thymocytes would not be so constrained.

The strong bias for CD4⁺ cells found in the transgenic thymus is also apparent in an analysis of peripheral T cells. As shown in Fig. 2b, lymph node cells from the transgenic mice are severely depleted of CD8⁺ cells. Most, if not all, of the few CD8⁺ T cells express $V\beta 3$ (not shown). Whether these cells express endogenous TCR as well is not known. The expression of endogenous TCR in these transgenic mice has not been deter-

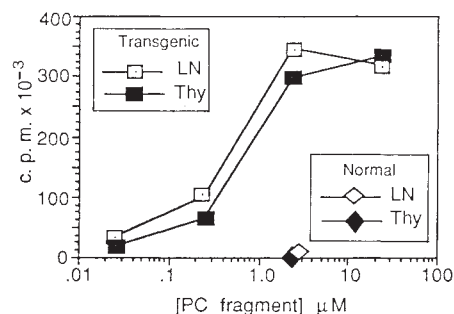


FIG. 4 Primary response of normal and $\alpha\beta$ transgenic cells to pigeon cytochrome c fragment. 5×10^4 thymocytes (closed symbols) or lymph node cells (open symbols) from a transgenic (boxes) or normal (diamonds) mouse were cultured in 96 well flat bottom tissue culture plates in the presence of 3×10^5 mitomycin-c treated autologous spleen cells and various concentrations of a synthetic carboxy-terminal fragment of pigeon cytochrome c, residues 88–104. After 48 h, cultures were pulsed with $1 \mu\text{Ci}$ [^3H] thymidine and harvested after an additional 16 h incubation. Data represent mean c.p.m. [^3H] thymidine incorporation of triplicate cultures. In the absence of pigeon cytochrome-c fragment all cultures showed a mean [^3H]thymidine incorporation of <400 c.p.m.

mined directly. If many of the transgenic TCR bearing cells also expressed random endogenous TCR on their surface, however, we would not have expected to see such a striking enrichment for CD4⁺ T cells.

In order to assess α and β transgene expression, thymocytes were stained with an anti-V β 3 monoclonal antibody KJ25 (ref. 24) and an anti-V α 11 monoclonal antibody 1F2 (S.J. & N.R.J.G., manuscript in preparation). This anti-V α 11 antibody binds T cells expressing V α 11.1-J α 84 and V α 11.2-J α 2B4 but its reactivity against other V α 11 family members and other V α 11-J α combinations is unknown. As shown in Fig. 3, expression of V β 3 or V α 11 in thymocytes of normal mice of these particular strains is low (3.7% and <0.5% respectively). In a transgenic littermate, however, 97% of thymocytes expressed V β 3 and 94% expressed V α 11 (Fig. 3). Similar to the finding with anti-CD3 (Fig. 1) approximately 80% of cells stained brightly with these reagents while 20% stained less brightly.

Because the mice analysed here express E^k, it is expected that cells bearing the transgenic TCR would be positively selected in the thymus and appear in the periphery. This is indeed the case. Less than 2% of lymph node cells from the normal mouse control express V β 3, and V α 11 expression was undetectable on these cells (Fig. 3). Conversely, 78% of lymph node cells from the transgenic mouse expressed V β 3 and 73% expressed V α 11 (Fig. 3). Given that these lymph nodes contained similar numbers of CD3⁺ cells (not shown), we conclude that virtually all the T cells in these mice express the cytochrome c specific TCR. In order to test this point and to assess whether the transgenic T cells were functional, we assayed for primary responses to a synthetic fragment of pigeon cytochrome c using autologous spleen cells as a source of antigen presenting cells. As shown in Fig. 4, both thymocytes and lymph node cells gave potent primary responses to the pigeon cytochrome c fragment. There was no detectable response from normal cells. The fact that the transgenic thymocytes were as responsive as peripheral T cells, in the absence of added factors, demonstrates that a large number were functionally, as well as phenotypically, mature.

The $\alpha\beta$ transgenic mice described in this report appear to be almost monoclonal in T-cell antigen specificity as well as CD4 phenotype. Surprisingly, the mice appear quite healthy. It remains to be determined whether these mice are indeed anergic to other antigens and environmental pathogens, and whether there are changes when the mice age. The changes noted in the thymus and periphery of these mice strongly support a positive selection process occurring during T-cell differentiation, linked to downregulation of CD8. It is not clear, however, whether the interaction of MHC and the receptor induces CD8 modulation or whether positive selection operates on transient randomly produced single positive populations. Finally, experiments to directly demonstrate the specificity for class II MHC molecules during positive selection in this system are in progress. □

23. Roehm, N., et al. *Cell* **38**, 577-584 (1984).
24. Pullen, A. M., Marrack, P. & Kappler, J. W. *Nature* **335**, 796-801 (1988).
25. Berg, L. et al. *Molec. cell. Biol.* **8**, 5459-5469 (1988).
26. Winoto, A. & Baltimore, D. *EMBO J.* **8**, 729-733 (1989).
27. Kaye, J. & Hedrick, S. M. *Nature* **336**, 580-583 (1988).
28. Krimpenfort, P. et al. *EMBO J.* **7**, 745-750 (1988).
29. Hogan, B., Costantini, F. & Lacy, E. In *Manipulating the Mouse Embryo* (Cold Spring Harbor Laboratory, New York, 1987).
30. Leo, O., Foo, M., Sachs, D. H., Samelson, L. E. & Bluestone, J. A. *Proc. natn. Acad. Sci. U.S.A.* **84**, 1374-1378 (1987).

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Peptide-dependent recognition of H-2K^b by alloreactive cytotoxic T lymphocytes

William R. Heath, Melissa E. Hurd, Francis R. Carbone & Linda A. Sherman

Department of Immunology, Scripps Clinic and Research Foundation, La Jolla, California 92037, USA

ANTIGEN-specific T lymphocytes appear to recognize foreign antigens in the form of peptide fragments presented within the antigen-binding groove of class I or class II molecules encoded by the major histocompatibility complex (MHC). Alloreactive T cells also show specificity for MHC molecules, and various reports suggest that residues of the MHC molecules constitute at least part of the ligand to which alloreactive T-cell receptors bind¹⁻⁴. The X-ray crystal structure of the human MHC class I molecule, HLA-A2⁵, has provided evidence to strengthen the argument that MHC-bound self-peptide⁶ might also contribute to such recognition⁷. We now provide direct evidence for this, showing that at least some alloreactive cytotoxic T lymphocyte clones recognize peptide fragments derived from cytoplasmic proteins. We reasoned that if self-peptides were involved in allorecognition, then the sequence of some of these peptides could vary between species, resulting in species-restricted distribution of the relevant ligand(s)⁸⁻¹¹. Several alloreactive cytotoxic T lymphocyte clones specific for H-2K^b, expressed by the murine cell line EL4, did not lyse a human-cell transfectant expressing the H-2K^b molecule (Jurkat-K^b cells). However, these clones were able to lyse Jurkat-K^b cells sensitized by preincubation with an EL4 cytoplasmic extract cleaved by cyanogen bromide. The sensitizing activity from this extract was destroyed by protease and appeared to be due to a peptide consisting of 10 to 15 amino acids.

To identify species-restricted recognition of allogeneic MHC molecules, eleven H-2K^b-specific alloreactive cytotoxic T lymphocyte (CTL) clones were tested for their capacity to recognize H-2K^b (K^b) expressed by the murine cell line EL4, but not by transfectants of the human cell line Jurkat. Three clones that expressed this specificity (EL4-K^b-specific) were identified. All three clones were isolated from the same responder, expressed the V β 8 segment of the T-cell antigen receptor, and showed similar target specificity, which indicated that they were probably sister clones. The target specificity of a representative EL4-K^b-specific clone (clone 35) is shown in Table 1 (experiment 1). Clone 52 is a representative cross-species reactive clone that was able to lyse both EL4 and Jurkat-K^b cells.

The specificity of the EL4-K^b-specific clones was determined by their ability to lyse the K^b-bearing cell lines C57BL/6.SV40 and B10.A(5R).SV40, but not the H-2^d cell line B10.D2.SV40 (Table 1, experiment 2). The specificity of the clones for K^b was further supported by their ability to release serine esterase in

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1. Babbitt, B., Allen, P. M., Matsueda, G., Haber, E. & Unanue, E. *Nature* **317**, 359-361 (1985).
2. Buus, S., Sette, A., Colon, S. M., Miles, C. & Grey, H. M. *Science* **235**, 1353-1358 (1987).
3. Zinkernagel, R. M. et al. *J. exp. Med.* **147**, 884-896 (1978).
4. Bevan, M. J. *Nature* **269**, 417-418 (1977).
5. Kisielow, P., Teh, H. S., Bluthmann, H. & von Boehmer, H. *Nature* **335**, 730-733 (1988).
6. Sha, W. C. et al. *Nature* **336**, 73-76 (1988).
7. Dembic, Z. et al. *Nature* **320**, 232-238 (1986).
8. Saito, T., Weiss, A., Miller, J., Norcross, M. A. & Germain, R. N. *Nature* **325**, 125-130 (1987).
9. Doyle, C. & Strominger, J. L. *Nature* **330**, 256-259 (1987).
10. Norment, A. M., Salter, R. D., Parham, P., Engelhard, V. H. & Littman, D. R. *Nature* **336**, 79-81 (1988).
11. Kronenberg, M., Siu, G., Hood, L. E. & Shastri, N. A. *Rev. Immun.* **4**, 529-591 (1986).
12. Swain, S. L. *Immun. Rev.* **74**, 129-142 (1983).
13. Saizawa, K., Rojo, J. & Janeway, C. A. Jr. *Nature* **328**, 260-263 (1987).
14. Marrack, P. et al. *J. exp. Med.* **158**, 1077-1091 (1983).
15. Veillette, A., Bookman, M. A., Horak, E. M. & Bolen, J. B. *Cell* **55**, 301-308 (1988).
16. Smith, L. *Nature* **326**, 798-800 (1987).
17. Fowlkes, B. J., Schwartz, R. H. & Pardoll, D. M. *Nature* **334**, 620-623 (1988).
18. Teh, H. S. et al. *Nature* **335**, 229-233 (1988).
19. Sha, W. C. et al. *Nature* **335**, 271-274 (1988).
20. Winoto, A. et al. *Nature* **324**, 679-682 (1986).
21. Fink, P. J., Matis, L. A., McElligott, D. L., Bookman, M. & Hedrick, S. M. *Nature* **321**, 219-226 (1986).
22. Hedrick, S. M. et al. *Science* **239**, 1541-1544 (1988).

TABLE 1 Target-cell specificity of K^b-specific alloreactive CTL clones

Target cell	Per cent specific lysis		Target cell	Per cent specific lysis	
	Clone 35	Clone 52		Clone 35	Clone 52
Experiment 1					
EL4	91	100	C57BL/6 con A	4	87
Jurkat-K ^b	7	70	B10.A(5R) con A	2	77
Jurkat-A2/K ^b	8	12	B10.D2 con A	2	2
			C57BL/6 LPS	19	92
			B10.A(5R) LPS	28	81
			B10.D2 LPS	0	4
Experiment 2					
C57BL/6.SV40 (K ^b , D ^b)	63	100	BALB/B LPS (K ^b , D ^b)	36	99
B10.A(5R).SV40 (K ^b , D ^d , L ^d)	100	99	BALB/c LPS (K ^d , D ^d , L ^d)	0	3
B10.D2.SV40 (K ^d , D ^d , L ^d)	6	1			
Experiment 3					
R8	82	92	C57BL/6 PEC	14	79
LB27.4	13	93	B10.A(5R) PEC	22	85
MC57	28	43	B10.D2 PEC	3	1

An EL4-K^b-specific CTL clone (clone 35) and a cross-species reactive K^b-specific CTL clone (clone 52) were analysed in several experiments for their capacity to lyse various K^b-expressing target cells. Target cells were labelled with ⁵¹Cr and specific lysis was measured in a 4-h ⁵¹Cr-release assay performed as previously described¹⁷. EL4 is a thymoma of C57BL/6 origin. Jurkat-K^b cells and Jurkat-A2/K^b cells refer to the human cell line Jurkat, transfected as previously described¹⁸ with either a plasmid constructed by transfer of an *Eco*R1 fragment containing the *H-2K^b* gene¹⁹ into pSV2neo, or with a plasmid containing an irrelevant chimaeric class I gene, *HLA-A2/K^b*, which encodes the $\alpha 1$ and $\alpha 2$ domains of HLA-A2 and the $\alpha 3$, transmembrane and cytoplasmic regions of K^b (ref. 18). Cell line R8 is derived from an Abelson leukaemia virus-transformed lymphosarcoma of (BALB/c $\times$ C57BL/6)F₁ origin²⁰. LB27.4 cells were produced by fusion of the BALB/c lymphoma A20.2J with T cell-depleted C57BL/10 spleen cells²¹. MC57 cells were derived from a C57BL/6 fibrosarcoma induced by 3-methylcholanthrene²². Various SV40-transformed cell lines were derived from spleen cell populations²³. Concanavalin A blasts were produced by stimulation of spleen cells (10^6 ml⁻¹) with concanavalin A (2.5 μ g ml⁻¹) for 3 days at 37 °C. Lipopolysaccharide blasts and thioglycollate-elicited peritoneal exudate cells were produced as previously described^{24,26}. EL4-K^b-specific CTL clones 6, 11 and 35 were derived from a (B10.D2 $\times$ B10.BR)F₁ mouse 10 days after priming with 2×10^7 EL4 cells. Cells were cloned by limiting dilution using irradiated B10.A(5R) (K^b, D^d, L^d) spleen-cell stimulators¹³. Cross-species reactive clones 52 and M37 were derived by limiting-dilution cloning of spleen cells from a naive or EL4 cell-primed B10.BR responder, respectively. CTL clones were maintained by weekly passage with irradiated C57BL/6 spleen-cell stimulators (3×10^6 ml⁻¹) in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 50 μ g ml⁻¹ gentamicin, 50 μ M 2-mercaptoethanol and 10% concanavalin A-stimulated rat spleen-cell supernatant²⁶. The effector-to-target ratios in each experiment were as follows: (1) Clone 35, 10:1; clone 52, 10:1. (2) Clone 35, 12:1; clone 52, 9:1. (3) Clone 35, 10:1; clone 52, 10:1. (4) Clone 35, 6:1; clone 52, 3:1. (5) Clone 35, 8:1; clone 52, 8:1. (6) Clone 35, 13:1; clone 52, 14:1.

response to affinity-purified K^b molecules (derived from EL4 cells) immobilized on a solid support¹³.

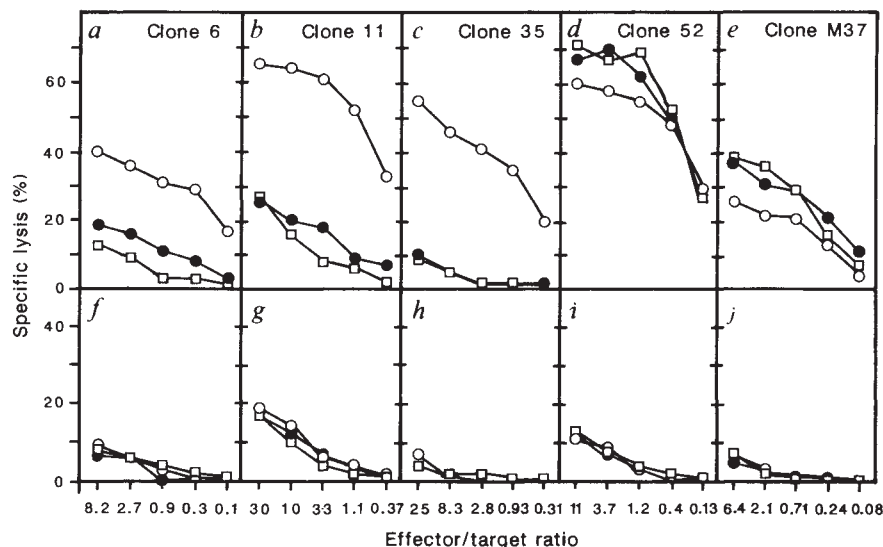
In an attempt to identify peptide antigen(s) that may be required for recognition of K^b by EL4-K^b-specific CTLs, a cytoplasmic extract was prepared from EL4 cells and subjected to cleavage by cyanogen bromide (CNBr). Jurkat-K^b cells were incubated with the CNBr-cleaved extract during ⁵¹Cr-labelling and examined for their susceptibility to lysis by the EL4-K^b-specific CTL clones 6, 11 and 35, and two cross-species reactive clones, 52 and M37 (Fig. 1, panels a-e). By contrast with clones 52 and M37, which lysed Jurkat-K^b-cells under all conditions,

the EL4-K^b-specific clones gave significant lysis of Jurkat-K^b cells exposed to CNBr-cleaved EL4-cell cytoplasm, but did not lyse cells exposed to uncleaved extract or to medium alone. Control Jurkat cells that were transfected with an irrelevant class I molecule, A2/K^b, were not sensitized by the CNBr-cleaved EL4-cell extract (Fig. 1, panels f-j).

The simplest explanation of these results is that the CNBr-cleaved EL4-cell extract contains specific peptide antigen(s) that are required for recognition of K^b by EL4-K^b-specific CTL clones. Another possibility, however, is that the EL4-K^b-specific CTL clones have a low affinity for K^b, and that the exposure

FIG. 1 Susceptibility of Jurkat-K^b target cells to lysis after sensitization with a CNBr-cleaved EL4-cell cytoplasmic extract. Jurkat-K^b cells (10^6) (panels a-e), or Jurkat-A2/K^b cells (10^6) (panels f-j) were incubated with 0.3 ml ⁵¹Cr (300 μ Ci) alone (●) or with 150 μ g (200 μ l) uncleaved (□) or CNBr-cleaved (○) EL4-cell cytoplasmic extract for 1.5 h at 37 °C.

METHODS. Cells were washed four times and then used as targets for lysis by the EL4-K^b-specific CTL clones 6, 11 and 35, and the cross-species reactive K^b-specific CTL clones 52 and M37. Cytoplasmic extract from EL4 cells was prepared from 10^9 cells. Cell pellets were frozen and thawed three times, then resuspended in 20 ml PBS. Nuclei were pelleted by centrifugation at 3,500g for 15 min at 4 °C, and the supernatant collected and centrifuged at 20,000g from 30 min at 4 °C. Supernatant from the second centrifugation was lyophilized then either cleaved with 0.5g CNBr in 70% formic acid as previously described²⁷, or left untreated. Extracts were resuspended in water to ~ 3.0 mg ml⁻¹ of protein, filtered and stored in 1 ml aliquots at -70 °C. On the day of assay, aliquots were thawed and diluted to ~ 750 μ g ml⁻¹ of protein in PBS.



of Jurkat-K^b cells to the CNBr-cleaved extract can non-specifically enhance the adhesion of Jurkat-K^b cells to CTL effectors. To test this possibility, a CNBr-cleaved extract of the human cell line JY was prepared. This extract did not sensitize Jurkat-K^b cells for lysis by EL4-K^b-specific clone 35 (Fig. 2a). Furthermore, we expected that if the adhesion properties of the target were altered, then there might be some increase in the lytic activity of the cross-species reactive clone M37, which lyses Jurkat-K^b cells poorly in comparison to clone 52. The activity of this potentially low-avidity clone, however, was not enhanced by sensitization of Jurkat-K^b cells. (Fig. 1, panels *d* and *e*). In addition, a K^b-restricted CTL clone that was specific for ovalbumin did not show enhanced lysis of Jurkat-K^b cells that were sensitized with CNBr-cleaved EL4-cell extract, either in the presence or absence of ovalbumin peptide (data not shown).

To determine whether the sensitizing activity of the CNBr-cleaved EL4-cell extract was due to a polypeptide, the preparation was exposed to pronase and then tested for its capacity

to sensitize Jurkat-K^b target cells (Fig. 2b). The sensitizing activity was destroyed under these conditions, indicating its protein origin.

To establish the size of the antigenic peptide(s) present in the CNBr-cleaved EL4-cell extract, the preparation was subjected to gel-filtration chromatography on Sephadex-G25. For all three EL4-K^b-specific CTL clones, Jurkat-K^b-sensitizing activity was identified in the included volume, migrating between markers of relative molecular mass (*M_r*) 1,300 and 1,700 (Fig. 3). Activity profiles for each clone indicated that all three clones recognize a 10–15-amino-acid peptide.

These results indicate that EL4-K^b-specific CTL clones recognize a peptide of 10–15 amino acids present in murine but not human cytoplasm. Further specificity analysis showed that not all murine cells were lysed by these clones (Table 1). Unlike control clones such as clone 52, which recognized K^b expressed by all of the targets that we tested, EL4-K^b-specific CTL clones lysed only a fraction (20–30%) of murine lipopolysaccharide (LPS) blasts and thioglycollate-elicited peritoneal exudate cells, and failed to lyse concanavalin A (con A) blasts. Most K^b-expressing cell lines were recognized by these clones, including EL4, R8, MC57 and SV40-transformed cell lines, although little reactivity was detected against the LB27.4 cell line. This indicated that expression of the peptide(s) recognized by these EL4-K^b-specific clones may be cell-type specific or tissue-specific. On the basis of these data, we could not attribute expression to a readily identifiable cell type; for example, T lymphoma cells (EL4), but not concanavalin-A-induced T-cell blasts, were susceptible to lysis by these CTL clones. Future experiments will attempt to determine whether this represents qualitative or quantitative differences between these cells. Cell-type specificity has been observed for both class I (ref. 14) and class II (refs 15 and 16)-specific alloreactive T cells. In view of the variability of lysis among murine cells the inability of these CTL clones to recognize Jurkat-K^b cells could be potentially attributable to cell-type specificity, rather than to species specificity.

The studies described here show that the ligand of alloreactive T cells can consist of a complex of peptide and allogeneic MHC molecule, analogous to the complex of peptide and syngeneic MHC molecule recognized by antigen-specific CTLs. Whether this is the general rule is yet to be determined. The experimental system that we have described is limited to identification of only

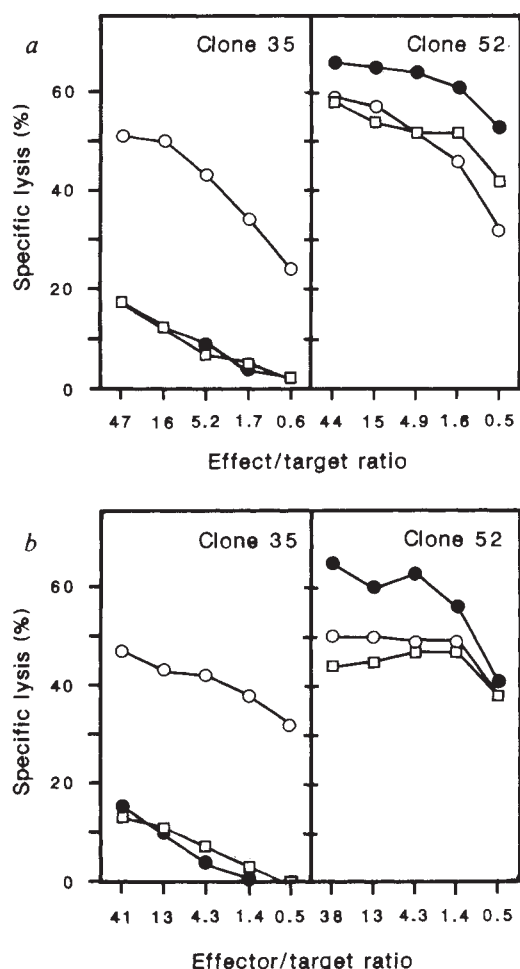


FIG. 2 *a*, Specificity of EL4-K^b-specific CTL clone 35 for EL4-derived extract. Jurkat-K^b-target cells (10^6) were exposed to ^{51}Cr for 1.5 h at 37 °C with medium alone (●), 150 μg (200 μl) CNBr-cleaved extract of EL4 cells (○), or 95 μg (200 μl) CNBr-cleaved extract of the human cell line JY (□), prepared as described in Fig. 1. Target cells were washed four times then examined for lysis by the EL4-K^b-specific CTL clone 35 or the cross-reactive K^b-specific CTL clone 52. *b*, Pronase destroys Jurkat-K^b sensitizing activity of the CNBr-cleaved EL4-cell extract. Jurkat-K^b-cells were exposed to ^{51}Cr for 1.5 h at 37 °C with medium alone (●), CNBr-cleaved EL4 extract (○) or extract treated with pronase (□), and examined for lysis by the EL4-K^b-specific CTL clone 35, or by the cross-species reactive clone 52. Jurkat-K^b-sensitization was as described in Fig. 1. Pronase digestion was performed by treating 225 μg (200 μl) CNBr-cleaved EL4-cell extract with pronase (1 U) immobilized on agarose (Sigma) for 3 h at 37 °C. Pronase was removed by centrifugation for 5 min at 400g.

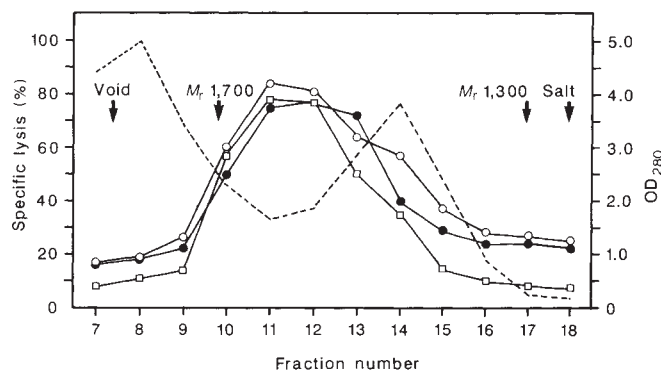


FIG. 3 Sephadex-G25 elution profile of the Jurkat-K^b-cell-sensitizing activity present in CNBr-cleaved EL4-cell extract. Jurkat-K^b cells were sensitized with CNBr-cleaved EL4 extract fractionated on a Sephadex-G25 column, then examined for recognition by EL4-K^b-specific CTL clones 6 (●), 11 (○) and 35 (□). CNBr-cleaved EL4-cell extract (1.8 mg in 0.6 ml) was loaded onto a 300 × 16 mm column of Sephadex-G25. Fractions (3 ml) were collected, lyophilized, and resuspended in 300 μl Hank's balanced salt solution. Each fraction (100 μl) was used to sensitize 10^6 Jurkat-K^b cells as described in Fig. 1. Arrows indicate the position of peak fractions containing blue dextran (void), a 15-residue peptide (*M_r* 1,700), a 10-residue peptide (*M_r* 1,300), and the salt peak, determined in separate experiments. Broken line indicates the optical density at 280 nm.

those peptides that differ in sequence between human and murine cells or that are cell-type specific in their expression. The use of cell lines that are phylogenetically more distant for transfection with K^b may provide a larger range of antigenic differences. Finally, the approach outlined here provides a strategy that may prove useful for defining the antigenic basis for other types of CTL responses, such as those to minor histocompatibility antigens, xenoantigens and tumour-associated antigens. □

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1. Parham, P. *et al. Nature* **325**, 625–628 (1987).
2. Clayberger, C. *et al. Nature* **330**, 763–765 (1987).
3. Song, E. S., Linsk, R., Olson, C. A., McMillan, M. & Goodenow, R. S. *Proc. natn. Acad. Sci. U.S.A.* **85**, 1927–1931 (1988).
4. Schneck, J., Maloy, W. L., Coligan, J. E. & Margulies, D. H. *Cell* **56**, 47–55 (1989).
5. Bjorkman, P. J. *et al. Nature* **329**, 512–518 (1987).
6. Buus, S., Sette, A., Colon, S. M. & Grey, H. M. *Science* **242**, 1045–1047 (1988).
7. Matzinger, P. & Bevan, M. J. *Cell. Immun.* **29**, 1–5 (1977).
8. Barbosa, J. A. *et al. Proc. natn. Acad. Sci. U.S.A.* **81**, 7549–7553 (1984).
9. Mentzer, S. J., Barbosa, J. A., Strominger, J. L., Biro, P. A. & Burakoff, S. J. *J. Immun.* **137**, 408–413 (1986).
10. Koller, T. D., Clayberger, C., Maryanski, J. L. & Krensky, A. M. *J. Immun.* **138**, 2044–2049 (1987).
11. Bernhard, E. J. *et al. J. Immun.* **139**, 3614–3621 (1987).
12. Bernhard, E. J., Le, A. I., Barbosa, J. A., Lacy, E. & Engelhard, V. H. *J. exp. Med.* **168**, 1157–1162 (1988).
13. Kane, K. P., Sherman, L. A. & Mescher, M. F. *J. Immun.* **142**, 4153–4160 (1989).
14. Manca, F. *et al. Transplantation* **36**, 670–674 (1983).
15. Minami, M., Kawasaki, H., Taira, S. & Nariuchi, H. *J. Immun.* **135**, 111–116 (1985).
16. Marrack, P. & Kappler, J. *Nature* **332**, 840–843 (1988).
17. Langlet, C., Neil, G. A. & Sherman, L. A. *J. Immun.* **139**, 3590–3596 (1987).
18. Irwin, M. J., Heath, W. R. & Sherman, L. A. *J. exp. Med.* (in the press).
19. Schultze, D. H. *et al. Molec. cell. Biol.* **3**, 750–755 (1983).
20. Ralph, P., Nakoinz, I. & Rashie, W. C. *Biochem. biophys. Res. Commun.* **61**, 1268–1275 (1974).
21. Kappler, J., White, J., Wegmann, D., Mustain, E. & Marrack, P. *Proc. natn. Acad. Sci. U.S.A.* **79**, 3604–3607 (1982).
22. Zinkernagel, R. M. *et al. J. exp. Med.* **148**, 592–606 (1978).
23. Pan, S., Wettstein, P. J. & Knowles, B. B. *J. Immun.* **128**, 243–246 (1982).
24. Manser, T. *J. Immun.* **139**, 234–238 (1987).
25. Gallily, R. & Feldman, M. *Immunology* **12**, 197–206 (1967).
26. Vitiello, A. & Sherman, L. A. *J. Immun.* **13**, 1635–1640 (1983).
27. Carbone, F. R., Moore, M. W., Sheil, J. M. & Bevan, M. J. *J. exp. Med.* **167**, 1767–1780 (1988).

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A macrophage Fc γ receptor and the mast cell receptor for IgE share an identical subunit

Chisei Ra, Marie-Hélène E. Jouvin, Ulrich Blank & Jean-Pierre Kinet*

Arthritis and Rheumatism Branch, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, Maryland 20892, USA

FC receptors for immunoglobulins are found on many immune cells and trigger essential functions of the immune defence system. With the exception of the high-affinity receptor for immunoglobulin E (Fc ϵ RI), these receptors were thought to consist of single polypeptides. Fc ϵ RI is a tetrameric complex of one α -subunit, one β -subunit and two γ -subunits. Here we report the cloning of a polypeptide identical to the γ -chains of Fc ϵ RI, from mouse macrophages that do not express this receptor. Biosynthetic labelling and gene transfer together show that these γ -chains associate with one of the macrophage receptors (Fc γ RIIa). The human homologue, Fc γ RIII (CD16), from natural killer cells is also expected to associate with γ -chains. It is possible that these γ -chains and the homologous ζ -chains of the T-cell antigen receptor belong to a new family of related proteins which share a common role in the signal transducing pathway.

Because Fc receptors are structurally and functionally related, the differences in their structural complexity are surprising:

TABLE 2 Expression of Fc γ RII on the surface of transfected COS-7 cells

Transfected genes	Binding of labelled antibody (fmol per 10 ⁶ cells)	Rosette forming cells (%)	FACS analysis % positive cells
Fc γ RIIa*	25 ± 10 (n=4)	0.2 ± 0.2 (n=11)	0.3 ± 0.2 (n=5)
Fc γ RIIa + γ *	301 ± 101 (n=4)	13.0 ± 7.5 (n=11)	8.7 ± 1.5 (n=5)
Fc γ RIIa + β + γ	ND	9.0 ± 3.5 (n=4)	6.0 ± 0.5 (n=2)
Fc γ RIIb ₂ *	1,700 ± 150 (n=4)	12.5 ± 10.5 (n=7)	ND
Fc γ RIIb ₂ + γ *	1,177 ± 342 (n=4)	17.5 ± 11.5 (n=7)	ND
γ *	1 ± 2 (n=3)	0 (n=8)	0.2 ± 0.3 (n=3)
Fc γ RIIa + ζ	ND	0 (n=1)	ND
Fc γ RIIa + β + ζ	ND	0 (n=1)	ND

Values are means ± standard deviation. The number of experiments is indicated in brackets. Binding experiments: Specific binding of ¹²⁵I-labelled antibody 2.4G2 per 10⁶ cells in each assay is given. Experiments of rosetting: The number of cells forming rosettes is expressed as the per cent of the total number of cells in the assay. When transfected cells were preincubated with antibody 2.4G2, the rosetting was completely inhibited (not shown). Fluorescence-activated cell sorting (FACS) experiments: The results are expressed as the per cent of specifically stained cells after subtraction of fluorescence measured when staining with non-biotinylated 2.4G2. In one of each experiment indicated by an asterisk, the relative amounts of specific transcripts for Fc γ RIIa and Fc γ RIIb₂ were tested in these transfectants by northern blotting and found to be equivalent. A comparable amount of γ -chain transcription was also found in γ -containing transfectants. When it was possible to compare them, the results from the rosetting assay were consistent with those obtained from the FACS analysis. This confirmed our previous assessment of the two assays⁸. ND, Not determined.

METHODS. Overlapping and similar DNA clones for γ -chains in macrophages were isolated using a rat mast cell probe for γ ³ to screen two different mouse libraries: a normal macrophage λ gt11 library from Clontech (California) and a λ ZAP library prepared from J774 mRNA as before⁸. The methods used to screen the libraries and to purify, subclone and sequence the positive clones have been described³. The following cDNA fragments were subcloned separately into the *Sma*I site of the SV40 promoter-driven expression vector pSVL (Pharmacia): the 834 bp *A*/wNI-*P*/fMI fragment of Fc γ RIIa, the 940 bp *B*/glI fragment of Fc γ RIIb₂, the 910 bp full-length cDNA of Fc ϵ RI β and the 389 bp *N*heI fragment of γ -chains from macrophage. The origin of the cDNAs for Fc γ RIIa, Fc γ RIIb₂ and Fc ϵ RI β has been given in Table 1. Complementary DNA for ζ -chain of the T cell antigen receptor was a gift from R. Klausner. COS-7 cells were transfected with 75–100 μ g DNA per 10⁷ cells by electroporation at 380 V and 25 μ F. After 48 h the transfected cells were tested for surface expression of Fc γ receptors. In binding experiments, cells were preincubated either with buffer or with a 20-fold excess of unlabelled 2.4G2 antibody for 45 min at room temperature and then incubated with a saturating amount of ¹²⁵I-labelled 2.4G2 antibody for 2 h at room temperature. After centrifugation through oil, cell-bound radioactivity was assessed as described⁴. For the IgG-rosetting assay, transfected cells were first incubated in the presence of buffer or with an excess of 2.4G2 antibody as specific inhibitor, and then with trinitrophenyl-derivatized red blood cells which had been preincubated with a saturating amount of rabbit anti-dinitrophenyl IgG (PharMingen, California). The rosetting assay was similarly performed on J774 cells as a positive control (not shown). For FACS analysis, cells were incubated either with uncoupled 2.4G2 antibody or with biotinylated 2.4G2 for 30 min on ice, then extensively washed and incubated with avidin-phycoerythrin for 30 min on ice, then again washed extensively. FACS analysis has been described⁸. Northern blots were prepared as described in the legend to Table 1, except that the same probe (corresponding to identical sequences between these receptors) was used to test for Fc γ RIIa and Fc γ RIIb₂.

* To whom correspondence should be addressed.

Fc ϵ RI has four subunits ($\alpha\beta\gamma_2$) and seven transmembrane domains, and the related Fc γ receptors have only one subunit and one transmembrane domain¹. This striking difference and the availability of DNA probes for β (ref. 2) and γ (ref. 3) led us to investigate whether subunits like β and γ could be associated with Fc receptors other than Fc ϵ RI. Because Fc ϵ RI is found exclusively on mast cells and basophils, we looked for messenger RNAs related to β and γ in cell lines that do not express this receptor. Table 1 shows that northern blotting detects γ -mRNA but not β -mRNA in 13 out of 18 cell lines. Furthermore, we detected systematic coexpression of transcripts for Fc γ RIIa (ref. 4) and the γ -subunit. Notably, the immunoglobulin-binding chain of Fc γ RIIa shares the most homology with the α -subunit from Fc ϵ RI (refs. 5-7). In particular, both proteins share eight consecutive amino acids, including an aspartic residue, in their respective transmembrane domains.

To assess the possible structural differences between the γ -chains from mast cells and their homologues in macrophages, two mouse libraries were prepared, one from normal macrophages and another from the cell line J774. Positive clones were isolated using a rat complementary DNA probe to γ ; six of these were characterized and were found to correspond in sequence to γ -cDNA clones isolated from the mast cell line PT18 (ref. 8). Therefore, at least the mRNAs for γ -chains in mouse mast cells and macrophages are identical. This supports our finding that γ -chains are encoded by a single gene²³.

We next investigated whether γ was associated with Fc γ RII. Using conditions that are known to maintain the non-covalent association of $\alpha\beta\gamma_2$ in Fc ϵ RI (ref. 9), biosynthetically labelled J774 cells were reacted with monoclonal antibodies 2.4G2 (specific for Fc γ RII) and the immune complexes were affinity-purified by a double immunospecific protocol. The

immunoprecipitated eluate was then analysed by gel electrophoresis and autoradiography. Figure 1 shows that Fc γ RII (relative molecular mass, 50,000) co-purifies with a peptide of lower molecular weight (M_r 20,000, or 20K) when the complexes are analysed under non-reducing conditions. When analysed quantitatively, all the counts associated with the 20K component (698 ³⁵S c.p.m.) in non-reducing conditions are recovered in the 10K component (623 ³⁵S c.p.m.) observed under reducing conditions (see legend to Fig. 2). These are the precise characteristics described for the γ -subunit of Fc ϵ RI (ref. 10). Because the monoclonal antibody we have used here does not distinguish between Fc γ RIIa and Fc γ RIIb₂ (ref. 4), both of which are present in J774 cells (ref. 4, and Table 1), it is not possible to assess from these experiments whether the γ -chains are associated with Fc γ RIIa, with Fc γ RIIb₂, or with both and so we cannot determine its stoichiometry.

Co-transfection of associated subunits has been shown to be a critical factor for efficient surface expression of various portions of multisubunit receptors such as the asialoglycoprotein receptor¹¹, the T-cell receptor¹², the CD3 complex¹³ and Fc ϵ RI (refs. 3, 14). For this reason, we compared the efficiency of surface expression of the respective receptors in COS-7 cells transfected with Fc γ RIIa or Fc γ RIIb₂. We confirmed an earlier report¹⁵ that expression of Fc γ RIIb₂ is much more efficient than expression of Fc γ RIIa (Table 2). When cells were transfected simultaneously with DNAs encoding γ -subunit and Fc γ RIIb₂, the efficiency of expression of this receptor was not increased. By contrast, co-transfection with the genes for γ and Fc γ RIIa dramatically increased the efficiency of surface expression of this receptor. Interestingly, the number of rosetting cells expressing Fc γ RIIb₂ or Fc γ RIIa + γ is comparable. The total number of receptors is higher in cells expressing Fc γ RIIb₂ than in those expressing Fc γ RIIa + γ , however, although transcription for each receptor was found equivalent (data not shown). This may

TABLE 1 Analysis by northern blotting

Cell types	Cell lines	Species	Transcripts identified				
			Fc ϵ RI			Fc γ RIIa	Fc γ RIIb
			α	β	γ		
1. Basophils	RBL	Rat	+	+	+	+	±
2. Mast cells	PT18	Mouse	+	+	+	+	+
3. Mastocytoma	P815	Mouse	-	-	+	+	+
4. Macrophages	J774	Mouse	-	-	+	+	+
5. Macrophages	P388	Mouse	-	-	+	+	+
6. Macrophages	PU5	Mouse	-	-	+	+	ND
7. Histiocytoma	212	Mouse	-	-	+	+	+
8. Myeloid cells	ABPL 2	Mouse	-	-	+	+	+
9. Pre-B cells	ABLS 103	Mouse	-	-	+	+	-
10. Pre-B cells	ABLS 140	Mouse	-	-	+	+	+
11. Plasmacytoma	TEPC 1173	Mouse	-	-	+	+	-
12. Plasmacytoma	TEPC 1196	Mouse	-	-	±	±	±
13. Plasmacytoma	ABPC 72	Mouse	-	-	+	+	+
14. T lymphoma	NTD	Rat	-	-	-	-	-
15. T lymphoma	S49	Mouse	-	-	-	-	+
16. T lymphoma	P1798	Mouse	-	-	±	±	-
17. T lymphoma	Baltelm 17	Mouse	-	-	+	+	+
18. Glial cells	C6	Rat	-	-	-	-	ND

The cell lines (numbers 1, 3-6, 15 and 18) were obtained from American Type Culture Collection and their RNA extracted as before⁵. The PT18 cell line (2) and the T lymphoma NTD (14) have been described²² and the other cell lines (7-13, 16, 17) were originally obtained from the Hazelton Laboratory (Rockville, Maryland) and their RNA supplied by K. Huppi. Northern blots and hybridization were as before⁵ and used the following cDNA probes: the 632 base-pair (bp) *Hind*III fragment specific for mouse Fc ϵ RI α , the full-length 910 bp fragment of mouse Fc ϵ RI β , the 418 bp *Hinc*II fragment specific for Fc ϵ RI γ , the 191 bp *Bam*HI fragment specific for mouse Fc γ RIIa (ref. 4), and the 187 bp *Bam*HI fragment specific for mouse Fc γ RIIb (ref. 4). The sequences of Fc ϵ RI α and γ probes described here were chosen so as to avoid any overlap with sequences of homologous portions of Fc γ RIIa and of T-cell receptor ζ -chains respectively. Cell lines (numbers 1-6, 14, 15 and 18) were also tested in non-stringent conditions (not shown) with the rat cDNA probes for α , β and γ of Fc ϵ RI (refs 2, 3 and 5). In these conditions, the results were similar to those described in the Table. The original cDNA clones of Fc γ RIIa and b₂ were a gift from J. Ravetch. Here we use Latin rather than Greek letters to define the different forms of Fc γ RII to avoid confusion with the subunits associated with Fc ϵ RI and Fc γ RII, for which we use Greek letters. The DNA sequences of the three subunits of mouse Fc ϵ RI are reported elsewhere⁸ and the cDNAs are available on request. ND, not determined.

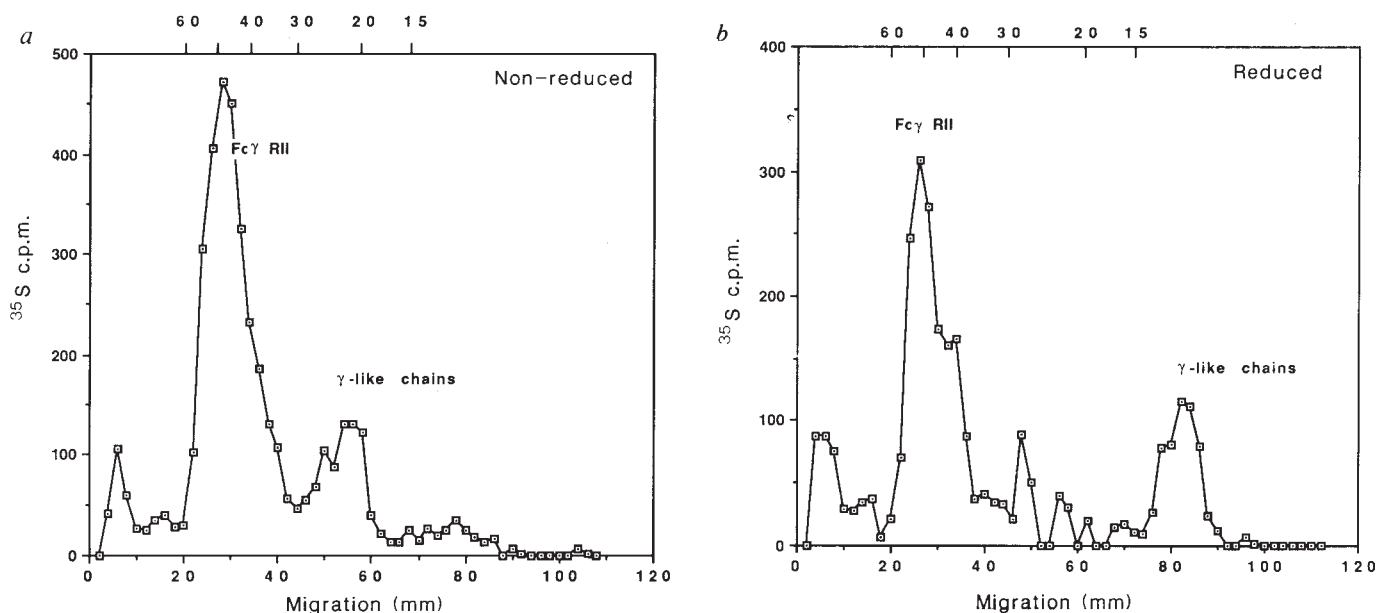


FIG. 1 Association of Fc γ RII with γ -chains. Fc γ RII receptors were purified from J774 cells labelled with ^{35}S -cysteine in 'non-dissociating' conditions and analysed by gel electrophoresis in *a*, the absence and *b*, the presence of reducing agent. M_r calibration (in thousands) is shown along the top in *a* and *b*.

METHODS. J774 cells were biosynthetically labelled with ^{35}S -cysteine by methods already described for rat basophilic leukaemia cells¹⁰. The cells were bound to monoclonal antibody 2.4G2 (ref. 4), which has been derivatized with trinitrophenyl and iodinated with ^{125}I -iodine²². Cells were then washed extensively to prevent low-affinity binding of monoclonal antibodies and solubilized at 5×10^7 cells per ml in borate-buffered saline containing 10 mM CHAPS (3-[(3-cholamidopropyl)dimethyl ammonio]-1-propane-sulphonate) and the same concentration of enzymatic inhibitors as before⁹. The trinitrophenyl-antibody-receptor complexes were reacted with control beads or with Protein A-Sepharose beads that had been coupled with rabbit IgG antindinitrophenyl antibodies²² and the beads were washed extensively with borate-buffered saline containing 10 mM CHAPS and 2 mM phospholipids extracted from a tumour of rat basophilic leukaemia cells⁹. The trinitrophenyl-antibody-receptor complexes were eluted in the same buffer but containing 5 mM dinitrophenyl- ϵ -amino caproate and immunoprecipitated

with goat anti-rat IgG (Jackson Immuno, Pennsylvania) and protein G beads (Pharmacia). After washing in 2 mM CHAPS, the precipitated material was counted for ^{125}I . The overall yield of antibody-bound receptors at this stage was estimated to be $\sim 10\%$. For example, in one of four experiments, 12×10^{12} receptor molecules were recovered from 7.5×10^7 cells, assuming divalent binding of 2.4G2 to the receptor. The pellet was then extracted in 1.25% SDS in gel sample buffer (containing 10% β -mercaptoethanol for reducing conditions) and applied to a 12.5% SDS-polyacrylamide gel. After electrophoresis, the gel was dried and autoradiographed. Each lane was cut into 2 mm slices for counting for ^{125}I and ^{35}S . The ^{35}S profiles shown here have been corrected for the overlapping ^{125}I counts arising from labelled antibodies. The eluate from the non-specific affinity column and the control immunoprecipitates were analysed and gave only a few counts at the top of the running gel. In non-reducing conditions, the ^{35}S associated with the γ -like peak gave 698 c.p.m. and the ^{125}I associated with the IgG peak gave 2,472 c.p.m. Under reducing conditions, there were 580 ^{35}S c.p.m. associated with the γ -like peak and 2,300 total ^{125}I counts associated with the heavy and light chains. The c.p.m. in the reduced γ -like peak (580 c.p.m.) can be corrected for the amount of ^{125}I loaded in the non-reduced lane for purposes of comparison (623 c.p.m.).

reflect the requirement imposed on Fc γ RIIa to assemble with γ -chains.

Taken together, these data demonstrate that Fc γ RIIa from macrophages share identical γ -chains with the mast cell receptor Fc ϵ RI. The γ -chains associated with Fc γ RIIa have probably not been detected before because of their easy dissociability, noted previously for Fc ϵ RI (refs 9, 16 and 17).

It is now established that human Fc γ RIII in natural killer cells^{18,19} is the human homologue of mouse Fc γ RIIa (ref. 20). With the α -chain of Fc ϵ RI, they each share the same eight consecutive residues in the transmembrane domain. Human

Fc γ RIII from natural killer cells will probably also be found to associate with γ -chains. Indeed, preliminary experiments using the polymerase chain reaction indicate that γ -chains are also present in human natural killer cells (M.-H.E.J. and J.-P.K., unpublished results).

Because γ -chains of Fc receptors and ζ -chains of T-cell receptors²¹ not only share homologous segments¹⁴, but are also located on the same mouse chromosome 1 (ref. 23), it is possible that they originate from a common ancestor. If so, their possible functional relationship may help to solve the most interesting question: how do all these receptors work? □

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- Kinet, J.-P. *Cell* **57**, 351–354 (1989).
- Kinet, J.-P. *et al. Proc. natn. Acad. Sci. U.S.A.* **85**, 6483–6487 (1988).
- Blank, U. *et al. Nature* **337**, 187–189 (1989).
- Ravetch, J. V. *et al. Science* **234**, 718–725 (1986).
- Kinet, J.-P., Metzger, H., Hakimi, J. & Kochan, J. *Biochemistry* **26**, 4605–4610 (1987).
- Kochan, J., Pettine, L. F., Hakimi, J., Kishi, K. & Kinet, J.-P. *Nucleic Acids Res.* **16**, 3584 (1988).
- Shimizu, A. *et al. Proc. natn. Acad. Sci. U.S.A.* **85**, 1907–1911 (1988).
- Ra, C., Jouvin, M.-H. & Kinet, J.-P. *J. biol. Chem.* **264**, 15323–15337 (1989).
- Kinet, J.-P. & Grasberger, B. In *Subcellular Biochemistry* (eds Harris, J. R. & Etemadi, A. H.) **14**, 321–337 (Plenum, New York, 1989).
- Perez-Montfort, R., Kinet, J.-P. & Metzger, H. *Biochemistry* **22**, 5722–5728 (1983).
- McPhaul, M. & Berg, P. *Proc. natn. Acad. Sci. U.S.A.* **83**, 8863–8867 (1986).

- Malissen, B., Steinmetz, M., McMillan, M., Pierres, M. & Hood, L. *Nature* **305**, 440–443 (1983).
- Berkhout, B., Alarcon, B. & Terhorst, C. *J. biol. Chem.* **263**, 8528–8536 (1988).
- Miller, L., Blank, U., Metzger, H. & Kinet, J.-P. *Science* **244**, 334–337 (1989).
- Weinshank, R. L., Luster, A. D. & Ravetch, J. V. *J. exp. Med.* **167**, 1909–1925 (1988).
- Rivnay, B., Wark, S. A. & Metzger, H. *Biochemistry* **21**, 6922–6927 (1982).
- Kinet, J.-P., Alcaraz, G., Leonard, A., Wark, S. & Metzger, H. *Biochemistry* **24**, 4117–4124 (1985).
- Ravetch, J. V. & Perussia, B. *J. exp. Med.* **170**, 481–497 (1989).
- Scallan, B. J. *et al. Proc. natn. Acad. Sci. U.S.A.* **86**, 5079–5083 (1989).
- Perussia, B. *et al. J. exp. Med.* **170**, 73–86 (1989).
- Weissman, A. M. *et al. Science* **239**, 1018–1021 (1988).
- Rivera, J., Kinet, J.-P., Kim, J., Pucillo, C. & Metzger, H. *Molec. Immun.* **25**, 647–661 (1988).
- Huppi, K., Siwarski, D., Mook, B. A. & Kinet, J.-P. *J. Immun.* (in the press).

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A cytosolic binding protein for the immunosuppressant FK506 has peptidyl-prolyl isomerase activity but is distinct from cyclophilin

John J. Siekierka, Shirley H. Y. Hung, Martin Poe, C. Shirley Lin & Nolan H. Sigal

Departments of Immunology Research and Enzymology, Merck, Sharp and Dohme Research Laboratories, PO Box 2000 Rahway, New Jersey 07065, USA

CYCLOSPORIN A and the newly discovered immunosuppressant, FK-506, are potent inhibitors of T cell activation¹. In addition to their clinical importance in the prevention of allograft rejection, cyclosporin A and FK-506 represent important reagents for the study of the molecular mechanisms of lymphocyte activation. Cyclosporin A, a cyclic undecapeptide and FK-506, a macrolide, although chemically distinct, inhibit similar lymphocyte activation responses^{1,2}. The earliest responses inhibited in the T cell seem to be the expression of early phase T cell-activation genes for interleukins 2, 3 and 4, granulocyte-macrophage colony stimulating factor and gamma interferon²⁻⁴. Although FK-506 and cyclosporin A seem to inhibit similar signal transduction processes, they do so by interacting with distinct cytosolic proteins^{5,6}. We report here the purification to homogeneity of a specific FK-506 binding protein that is distinct from the cyclosporin A-binding protein, cyclophilin^{7,8}. In addition, we show that this FK-506 binding protein, like cyclophilin, has peptidyl-prolyl isomerase activity^{9,10}.

In a preliminary study⁶, we found that the potent immunosuppressive agent, FK-506, was rapidly taken up by T cells and was largely associated with a low molecular weight cytoplasmic component. Using cytosol prepared from the JURKAT T-cell

line, we purified to apparent homogeneity a specific FK-506 binding protein (FKBP). This binding protein represents ~0.4% of the total cytoplasmic protein in JURKAT cells (Table 1), a value similar to that reported for cyclophilin (0.1–0.4%; ref. 11). A summary of the purification procedure is given in Table 1. Electrophoresis (Fig. 1, lane 2) of the purified FKBP reveals a single homogeneous band with a relative molecular mass of 10,000–11,000 (10–11K), in agreement with the value previously obtained by HPLC gel filtration⁶ and lower than the molecular mass of cyclophilin (Fig. 1, lane 3 and ref. 11). Purified FKBP had a specific activity of 26 μ g of FK-506 bound per mg of protein, with protein determined by the Bradford assay¹². The theoretical value expected for a 1:1 molar complex of FK-506 to FKBP is 73–80 μ g mg⁻¹; the Bradford protein assay, however, seems to overestimate the actual quantity of FKBP protein by approximately threefold when compared with other methods

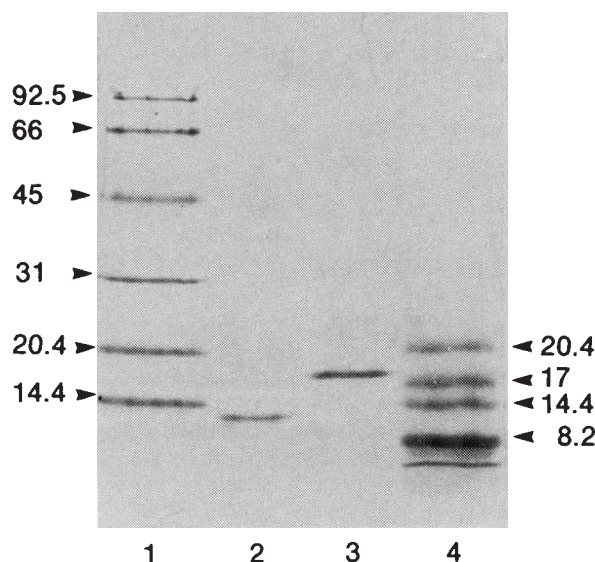


FIG 1 SDS-PAGE of purified FK-506 binding protein and calf thymus cyclophilin. Lane 1, protein standard; lane 2, FK-506 binding protein; lane 3, calf thymus cyclophilin; lane 4, low molecular weight standards (Enprotech). Molecular weights are shown in thousands.

METHODS. HPLC CM 300-purified FKBP (3 μ g) and calf thymus cyclophilin (3 μ g)¹¹ were electrophoresed on a 12% polyacrylamide gel using a Mini-Protein II slab gel apparatus (Bio-Rad). Gels were stained with Coomassie blue.

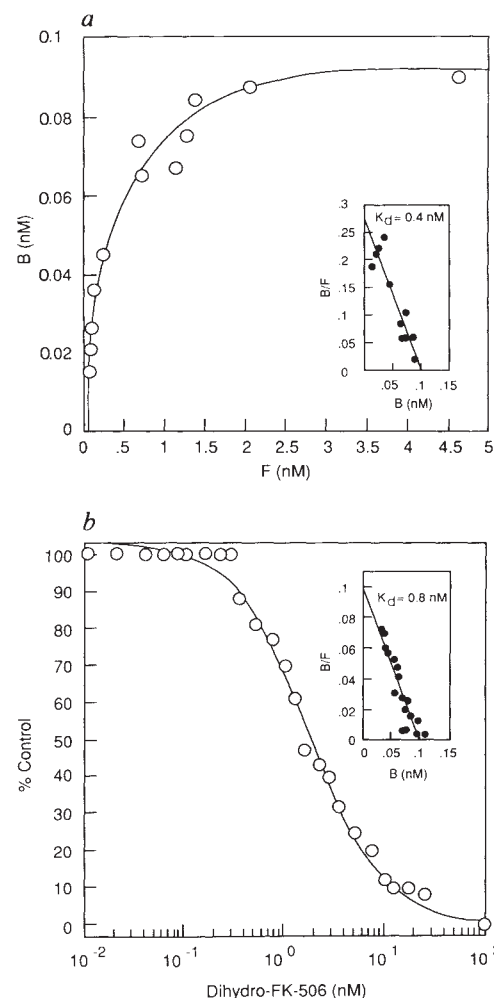
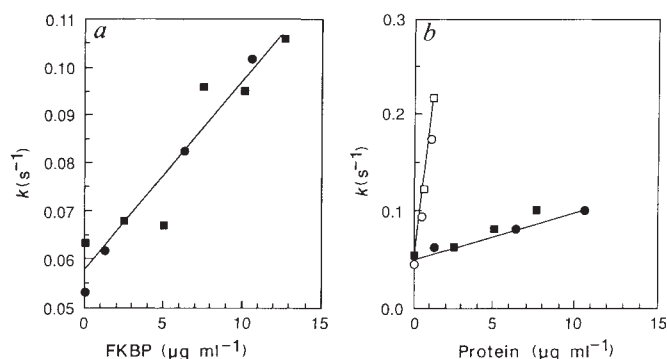


FIG. 2 Binding of [³H]dihydroFK-506 to the cytosolic binding protein. *a*, Direct binding of [³H]dihydroFK-506 to FKBP. *b*, Competitive binding of [³H]dihydroFK-506 with unlabelled dihydroFK-506. Insets illustrate the data plotted in the Scatchard format. *B*, Concentration of [³H]dihydroFK-506 specifically bound; *F*, that which is free.

METHODS. Assays were conducted in 96-well microtitre plates previously treated with the siliconizing agent Sigmacote (Sigma). The assays contained 20 mM sodium phosphate (pH 7.2), 0.5% BSA, 1.25 ng ml⁻¹ purified FK-506 binding protein (step V) and the amounts of [³H]dihydroFK-506 indicated. The mixtures were incubated for 20 min at 25 °C and assayed by the LH-20 assay as described (Table 1). Competitive binding assays were the same as direct binding, except that assays contained 1.8 nM [³H]dihydroFK-506 and the indicated amounts of unlabelled competitor. The binding data were analysed using the LIGAND program¹³ and the data plotted in the Scatchard format.

FIG. 3 FKBP peptidyl-prolyl *cis-trans* isomerase activity (PPIase). *a*, PPIase activity as a function of FKBP concentration. Different symbols represent the results of experiments with two different preparations of FKBP (step V). *b*, Comparison of PPIase activity associated with cyclophilin (open symbols) and FKBP (Step V) (closed symbols). Symbols as above.

METHODS. PPIase activity was assayed essentially as described¹⁰. The assay measures the '*cis*' to '*trans*' isomerization of the proline-alanine peptide bond in the peptide, *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide. The '*trans*' form of the peptide is readily cleaved by chymotrypsin with the release of *p*-nitroanilide which is quantitated spectrophotometrically at 405 nm. At equilibrium, 88% of the peptide is present as the '*trans*' form. The remaining 12% of the peptide present in the '*cis*' form is cleaved on enzymatic conversion to the '*trans*' form. Reactions (1.0 ml) were at 25 °C and contained 0.1 mM substrate (Sigma) added as a 30 μ l aliquot of a 2.1 mM stock in dimethyl sulphoxide, 100 mM Tris-HCl (pH 7.8), cyclophilin or FKBP (step V, Table 1) and, where indicated, either CsA or FK-506. After 1 min, 30 μ l of 2 mg ml⁻¹ chymotrypsin (Sigma) in 100 mM Tris-HCl (pH 7.8) was added. After mixing, the increase in absorbance at 405 nm was measured in a Beckman DU68 spectrophotometer at 3 s intervals. The log



of the difference between absorbance at steady state and absorbance at time t was plotted against time. The first order rate constant, k (s⁻¹), is calculated from the slope of the resulting straight line.

such as ultraviolet absorbance and amino-acid analysis (D. Boulton *et al.*, manuscript in preparation). A similar observation has been reported for cyclophilin¹¹. Correcting the binding data for this discrepancy would yield a specific activity of 78 μ g mg⁻¹, indicating the formation of a 1:1 complex of FK-506 and FKBP. Preliminary sequence analysis revealed a single N-terminus, indicating that the protein represents a single homogeneous species. In addition, no homology between this sequence and that of cyclophilin was observed; FKBP does not, therefore, seem to be a processed form of cyclophilin (D. Boulton *et al.*, manuscript in preparation).

Binding of [³H]dihydroFK-506 to FKBP is saturable and of high affinity (Fig. 2*a*). Cyclosporin A (CsA) at concentrations ≤ 1 μ M, failed to compete with FK-506 for binding (data not shown). Scatchard analysis of the direct binding data yields a dissociation constant (K_d) of 0.4 nM for the interaction (Fig. 2*a*, inset). In addition, competition binding data were analysed

using the LIGAND computer program¹³. The data best fits a single-site model with a K_d of 0.8 nM (Fig. 2*b*, inset), consistent with the direct binding approach. The K_d values obtained by these two approaches are similar to the concentration of dihydroFK-506 that caused 50% inhibition of interleukin-2 (IL-2) secretion in JURKAT cells stimulated with phorbol myristate acetate /ionomycin (1.1 nM; ref. 6). These results are consistent with the notion that the immunosuppressive activity of FK-506 is mediated by its interaction with this binding protein.

It has recently been demonstrated^{9,10} that cyclophilin is identical to peptidyl-prolyl isomerase (PPIase), an enzyme that catalyses the *cis-trans* isomerization of proline residues in proteins and peptides. In addition, CsA was found to inhibit cyclophilin PPIase activity at concentrations similar to those that inhibit T cell activation. The catalysis of proline *cis-trans* isomerization is believed to be important in folding of proteins into their native conformations. It has also been proposed that

TABLE 1 Purification of FKBP from JURKAT Cells

Purification step	Volume (ml)	Protein (mg ml ⁻¹)	Specific activity (μ g FK-506 bound per mg protein)	Total binding capacity (μ g FK-506)	Recovery (%)
I S-100	127	8.06	0.076	77.8	100
II 56 °C, 20 min	114	2.59	0.17	50.2	65
III Affigel Blue	2.2	4.59	3.63	39.5	51
IV TSK-125 HPLC	2.2	0.64	12.90	18.2	23
V CM 300 HPLC	0.77	0.17	25.66	3.4	4.3

FK-506 and [³H]dihydroFK-506 (specific activity, 49 mCi mg⁻¹) were prepared as previously described^{2,6}. Binding of [³H]dihydroFK-506 to JURKAT protein fractions was assayed by the LH-20 assay⁷ with 30 nM [³H]dihydroFK-506 and 0.1–10 μ g protein. Bound [³H]dihydroFK-506 was separated from free [³H]dihydroFK-506 by chromatography on individual 2 ml Sephadex LH-20 columns (Pharmacia) (ref. 7). Samples (500 μ l) were mixed with 3.0 ml Aquasol-2 (New England Nuclear, Boston) and counted in a LS-9000 scintillation counter (Beckman Instruments) at 50% efficiency. JURKAT cells (clone 77. 6.8, a gift from Dr Kendall Smith) were cultured in RPMI 1640 media (Cellgro) containing 1% penicillin/streptomycin, 1 mM glutamine (both from Gibco Laboratories) and 10% heat-inactivated fetal calf serum (Hazleton Biologics). Approximately 70×10^9 cells were washed twice with 150 ml PBS and pelleted by low-speed centrifugation. The cells were disrupted by Dounce homogenization in 4 volumes of buffer containing 10 mM Tris-HCl (pH 7.5), 100 mM KCl, 5 mM 2-mercaptoethanol, 0.5 mM PMSF and 1 mM EDTA, and centrifuged at 500g for 20 min. After centrifugation for 1 h at 100,000g, the supernatant was heated for 20 min at 56 °C, precipitated protein removed by centrifugation and the resulting supernatant concentrated in an Amicon ultrafiltration cell with a YM5 membrane (Amicon). The same procedure was used to concentrate protein in subsequent steps. The concentrated protein was dialysed against buffer containing 5 mM 2-mercaptoethanol, 0.5 mM PMSF, 1 mM EDTA and 10 mM KH₂PO₄ (pH 7.2) and applied to a 1.5 $\times$ 40 cm Affigel Blue column (Bio-Rad) equilibrated with the same buffer. Protein was eluted with a linear gradient (180 ml total) of 10–200 mM KH₂PO₄ containing 5 mM 2-mercaptoethanol, 0.5 mM PMSF and 1 mM EDTA. Fractions (2.5 ml) were collected and assayed for FK-506 binding. The peak of activity (at ~ 50 mM KH₂PO₄) was dialysed against buffer containing 20 mM NaH₂PO₄, 50 mM Na₂SO₄ (pH 6.8), 5 mM 2-mercaptoethanol, 0.5 mM PMSF and 1 mM EDTA, and concentrated and fractionated by HPLC on a TSK-125 gel filtration column (21 $\times$ 600 mm, Bio-Rad). Fractions (4 ml) were assayed and the peak of activity pooled and dialysed against buffer containing 5 mM KH₂PO₄ (pH 6.8), 5 mM 2-mercaptoethanol, 0.5 mM PMSF and 1 mM EDTA. The protein was concentrated and fractionated by weak ion exchange on a SynChropak CM 300 column (4.6 $\times$ 250 mm, Rainin Instruments). Protein was eluted isocratically at a flow rate of 0.3 ml min⁻¹ with buffer of the same composition as the dialysis buffer. Fractions (0.3 ml) were assayed and the peak of activity pooled and concentrated. The purified protein was stored at 0 °C for short periods and -80 °C for extended periods. Protein was determined by the Bradford assay (Bio-Rad) with IgG as the standard.

proline *cis-trans* isomerization may be involved in regulating signal transduction by membrane receptors and membrane transport proteins¹⁴. It is interesting, therefore that the *ninaA* gene product, which is required for visual transduction in *Drosophila*, has extensive sequence homology to cyclophilin¹⁵. These observations indicate that cyclophilin may have a regulatory role in T cell signal transduction through its PPIase activity. We have found that FKBP, like cyclophilin, catalyses the *cis-trans* isomerization of the Ala-Pro bond in the peptide, *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Fig. 3a). Cyclophilin, under similar conditions, exhibits a 25-fold greater activity per mg of protein than FKBP (Fig. 3b). The lower PPIase activity associated with FKBP is not due to heat inactivation of the catalytic site during purification because FKBP prepared without the heat treatment step had essentially the same activity before and after heat treatment (data not shown); it may, therefore, reflect a more stringent substrate requirement. The physiologically relevant substrates for both cyclophilin and FKBP have not yet been identified. FKBP PPIase activity is specifically inhibited by FK-506 (Fig. 4a), whereas CsA at a concentration of 1 μ M had no effect. These data rule out the possibility that the FKBP activity is due to contamination by cyclophilin. The large amount of FKBP used in these assays ($>7 \mu\text{g ml}^{-1}$; 0.7 μM) precludes a valid comparison of the concentration of FK-506 needed for half-maximal PPIase inhibition (IC_{50}) with the IC_{50} for FK-506 in the JURKAT IL-2 assay. Cyclosporin A is a potent inhibitor of cyclophilin PPIase activity (Fig. 4b),

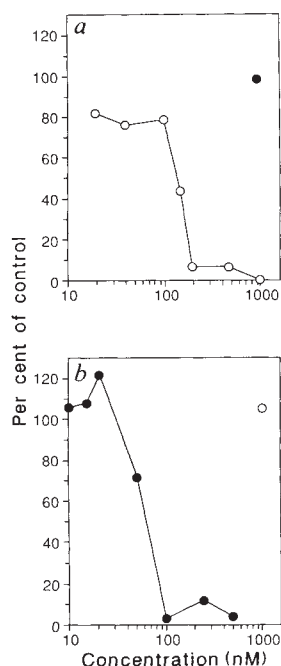


FIG. 4 Effect of FK-506 and CsA on FKBP and cyclophilin PPIase activity. *a*, Inhibition of FKBP (step IV) PPIase activity by FK-506, open circles; CsA, closed circle. *b*, Inhibition of cyclophilin PPIase activity by CsA, closed circles; FK-506, open circle.

METHODS. PPIase activity was assayed as described for Fig. 3. Stock solutions of FK-506 and CsA (1 mg ml^{-1}) were prepared in ethanol and diluted with assay buffer to the concentrations indicated. Step IV (Table I) FKBP was present at $31.4 \mu\text{g ml}^{-1}$ in *a*. Step IV FKBP was used to facilitate the quantitation of FKBP PPIase activity at lower enzyme concentrations. Cyclophilin was present at $0.44 \mu\text{g ml}^{-1}$ in *b*. Addition of ethanol had no effect on the PPIase assays.

whereas FK-506 at a concentration of 1 μM gave no inhibition. These data further support our contention that FKBP and cyclophilin are distinct proteins with distinct ligand specificities.

The isolation of a unique protein (FKBP) that binds the potent immunosuppressive agent, FK-506, provides further insight into the molecular basis of T cell activation and immunosuppression. Although FKBP is distinct from cyclophilin by a number of criteria, the two proteins do share several common properties. Both proteins are abundant, are found mainly in the cytoplasmic compartment of cells, have low molecular weights, bind immunosuppressive ligands and exhibit PPIase activity that is specifically inhibited by ligand binding.

Both the immunosuppressive agents FK-506 and CsA share a number of common cell-biological properties. In both T and B lymphocytes, we and others^{1,16,17} have been unable to distinguish between the effects of FK-506 and CsA when the cells have been triggered with pharmacological agents or cell-surface molecules that use different intracellular activation pathways. In particular, the ability of either FK-506 or CsA to inhibit these events was found to vary in the same way, according to the mode of cellular activation. In general, only activation pathways that caused a measurable rise in intracellular Ca^{2+} were FK-506/CsA-sensitive^{16,18}. For example, FK-506 and CsA inhibited activation through the CD3 pathway, but not proliferation induced through the CD28 pathway, in human lymphocytes¹⁶. PPIases may interact with such Ca^{2+} -dependent signal transduction pathways by influencing the conformation of protein kinases, ion channels or transcription factors in lymphocytes. The finding that FKBP, like cyclophilin, possesses PPIase activity, is evidence that the PPIase activity of both proteins plays a critical part in T cell activation. The molecular mechanism(s) that involves such an isomerase activity in lymphocyte-specific signal transduction events remains to be discovered. There is genetic evidence that cyclophilin-like molecules participate in a defined signal transduction pathway (visual transduction) and that a photoreceptor cell-specific form of this enzyme may exist¹⁵. We hypothesize that there are specific substrates or unique forms of the enzyme in lymphocytes which explain the selective sensitivity of this cell type to these immunosuppressive agents. The potential existence of a cyclophilin gene family^{15,19,20} supports the hypothesis that cell-specific forms of cyclophilin (and perhaps FKBP) may exist. These observations lead us to suggest that FKBP and cyclophilin, through their peptidyl-prolyl isomerase activity, may be important in T cell activation. □

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1. Sawada, S., Suzuki, G., Kawase, Y. & Takaku, F. *J. Immun.* **139**, 1797–1803 (1987).
2. Tocci, M. J. et al. *J. Immun.* **143**, 718–726 (1989).
3. Kronke, M. et al. *Proc. natn. Acad. Sci. U.S.A.* **81**, 5214–5218 (1984).
4. Elliott, J. F. et al. *Science* **226**, 1439–1441 (1984).
5. Warty, V. et al. *Transplantation* **46**, 453–455 (1988).
6. Siekierka, J. J., Staruch, M. J., Hung, S. H. Y. & Sigal, N. H. *J. Immun.* **143**, 158–1583 (1989).
7. Handschumacher, R. E., Harding, M. W., Rice, J., Druggie, R. J. & Speicher, D. W. *Science* **226**, 544–547 (1984).
8. Harding, M. W. & Handschumacher, R. E. *Transplantation* **46**, 29S–35S (1988).
9. Takahashi, N., Hagano, T. & Suzuki, M. *Nature* **337**, 473–475 (1989).
10. Fischer, G., Wittmann-Liebold, B., Lang, K., Kiehlhauer, T. & Schmidt, F. X. *Nature* **337**, 476–478 (1989).
11. Harding, M. W., Handschumacher, R. E. & Speicher, D. W. *J. biol. Chem.* **261**, 8547–8555 (1986).
12. Bradford, M. M. *Analyt. Biochem.* **72**, 248–254 (1976).
13. Munson, P. J. & Rodbard, D. *Analyt. Biochem.* **107**, 220–239 (1980).
14. Brandt, C. L. & Deber, C. M. *Proc. natn. Acad. Sci. U.S.A.* **83**, 917–921 (1986).
15. Shieh, B.-H., Starnes, M. A., Seavello, S., Harris, G. L. & Zuker, C. S. *Nature* **338**, 67–70 (1989).
16. Lin, C. S. et al. *J. Immun.* (submitted).
17. Wicker, L. et al. *J. Immun.* (submitted).
18. June, C. H., Ledbetter, J. A., Gillespie, M. M., Lindsten, T. & Thompson, C. B. *Molec. cell. Biol.* **7**, 4472–4481 (1987).
19. Haendler, B., Hofer-Warbinek, R. & Hofer, E. *EMBO J.* **6**, 947–950 (1987).
20. Danielson, P. E. et al. *DNA* **7**, 261–267 (1988).

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A receptor for the immuno-suppressant FK506 is a *cis-trans* peptidyl-prolyl isomerase

Matthew W. Harding, Andrzej Galat,[†]
David E. Uehling[†] & Stuart L. Schreiber[†]

Division of Organ Transplantation and Immunology, Department of Surgery, Yale University School of Medicine, 310 Cedar St, New Haven, Connecticut 06510, USA

[†] Department of Chemistry, Harvard University, 12 Oxford Street, Cambridge, Massachusetts 02138, USA

THE structurally novel macrolide FK506 (refs 1,2) has recently been demonstrated to have potent immunosuppressive activity³⁻⁷ at concentrations several hundredfold lower than cyclosporin A (CsA). Cyclosporin A, a cyclic peptide, has found widespread clinical use in the prevention of graft rejection following bone marrow and organ transplantation⁸. The mechanisms of immunosuppression mediated by FK506 and CsA appear to be remarkably similar, suggesting that these unrelated structures act on a common receptor or on similar molecular targets, perhaps the CsA receptor, cyclophilin⁹⁻¹¹, which has recently been shown by Fischer *et al.*¹² and Takahashi *et al.*¹³ to have *cis-trans* peptidyl-prolyl isomerase activity. We have prepared an FK506 affinity matrix and purified a binding protein for FK506 from bovine thymus and from human spleen. This FK506-binding protein (FKBP) has a relative molecular mass (M_r) of ~14,000(14K), a pI of 8.8-8.9, and does not cross-react with antisera against cyclophilin. The first 40 N-terminal residues of the bovine and 16 residues of the human FKBP were determined; the 16-residue fragments are identical to each other and unrelated to any known sequences. This protein catalyses the *cis-trans* isomerization of the proline amide in a tetrapeptide substrate and FK506 inhibits the action of this new isomerase. The FKBP and cyclophilin appear to be members of an emerging class of novel proteins that regulate T cell activation and other metabolic processes, perhaps by the recognition (and possibly the isomerization) of proline-containing epitopes in target proteins.

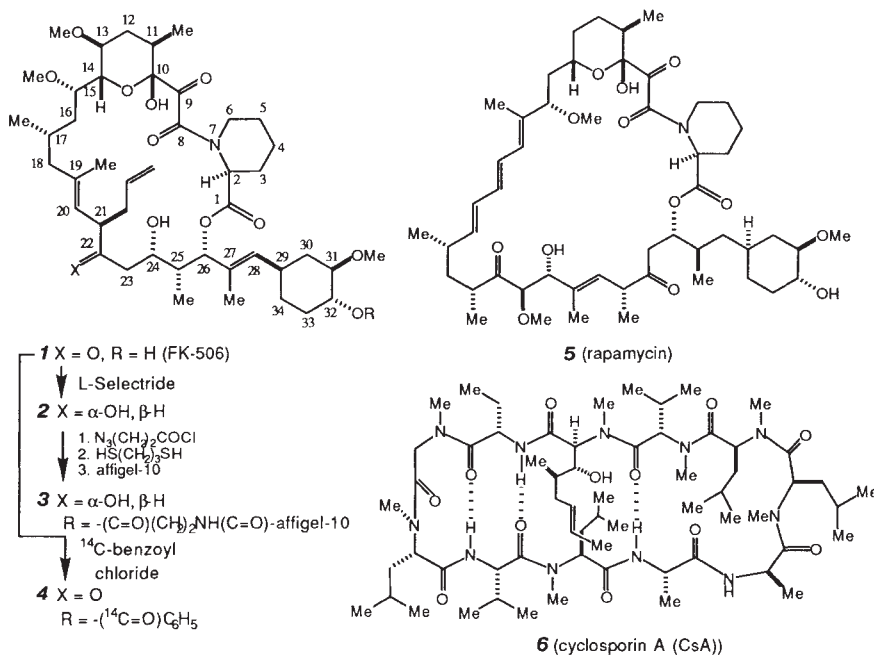
FK506 (Fig. 1;1) and CsA (Fig. 1;6) affinity matrices were prepared using an FK506 amino derivative, synthesized as described in the legend to Fig. 1, and an 8'-ornithine CsA derivative, respectively. When cytosol extracts of bovine thymus and of human spleen were adsorbed onto the FK506 matrix and the column eluted with FK506, a single protein of ~14K was obtained (Fig. 2, lanes 1 and 5). FK506 also displaced a 14K protein from the FK506 matrix in experiments with cytosol extracts of bovine kidney, human and murine liver, and EL4 cells. Analogous experiments with the CsA matrix eluted with CsA produced bovine and human cyclophilins, a 14K bovine protein, and human proteins of M_r s 15K, 36K and 40K (Fig. 2, lanes 2 and 6). No proteins were retained in a control experiment with a non-derivatized matrix (Affigel-10 capped with ethanolamine). The similarity of FK506 and CsA binding proteins was analysed by immunoblotting. Affinity-purified rabbit anti-cyclophilin IgG reacted strongly with bovine and human cyclophilins and the bovine CsA displaced 14K protein (Fig. 2, lanes 4 and 8), but did not react with the bovine and human FK506-displaced 14K proteins (Fig. 2, lanes 3 and 7). These data indicate that the FKBP is antigenically unrelated to cyclophilin. The 15K, 36K and 40 K human spleen proteins were not recognized by the antiserum (Fig. 2, lane 8).

The bovine FKBP was found to have an N-terminal sequence of H₂N-Gly-Val-Gln-Val-Glu-Thr-Ile-Ser-Pro-Gly-Asp-Gly-Arg-Thr-Phe-Pro-Lys-Arg-Gly-Gln-Thr-Cys-Val-Val-His-Tyr-Thr-Gly-Met-Leu-Glu-Asp-Gly-Lys-Lys-Phe-Asp-Ser-Ser-Arg-. The first 16 residues of the human protein were determined and found to be identical to the corresponding bovine sequence. Isoelectric focusing of the bovine and human FKBP revealed a pI of 8.8-8.9. Comparison of the N-terminal 40-amino-acid sequence (*vide supra*) with the NBRF protein sequence database (release 20.0, 3/89) and of the human and murine complementary DNA sequences corresponding to amino acids 7-29 (unpublished results of R. F. Standaert, G. L. Verdine and S.L.S.) with the GenBank (release 59.0, 3/89) and EMBL (release 18.0, 2/89) databases failed to identify any significant similarity to known sequences. These FK506-binding proteins appear to represent a previously unidentified class of conserved proteins.

In order to examine drug binding to the native protein, a chromatographic procedure (without use of the affinity reagent; see Fig. 1;4) was developed that gave rise to homogeneous drug-free bovine and human FKBP (Fig. 2c). The identity of

FIG. 1 Structure of FK506, 1; rapamycin, 5; cyclosporin A, 6; the reaction sequence for the synthesis of FK506-affigel, 3; and [¹⁴C]benzoyl derivatives, 4.

METHODS. The synthesis of the FK506-affinity reagent began with the stereoselective reduction of the C(22) ketone in 1 with L-Selectride²⁰ and generated the tetraol, 2. Selective acylation of the C(32) hydroxyl with β -azidopropionic acid was achieved by the conversion of this reagent to the corresponding acid chloride followed by a reaction with 2. Chemoselective reduction of the azide proceeded under the conditions of Knowles²¹ and generated an FK506-amino derivative (structure not shown). For preparation of the affinity matrices, the FK506-amino and 8'-ornithine CsA derivatives were diluted in ethanol to 1 mg ml⁻¹ and mixed with an equal volume of Affigel 10 (BioRad) for 4 h at room temperature with 0.1 N NaHCO₃. Unbound drug was recovered by washing the resins with isopropanol. Unreacted groups were blocked by shaking for 4 h with 200 mM ethanolamine-HCl in 0.1 N NaHCO₃. TLC analysis of wash fractions indicated >85% coupling of FK506. The efficiency of CsA coupling was not quantitated but was presumed to be similar. 32-[1-¹⁴C]-labelled FK506 analogue 4 was prepared by selective benzoylation of 1.

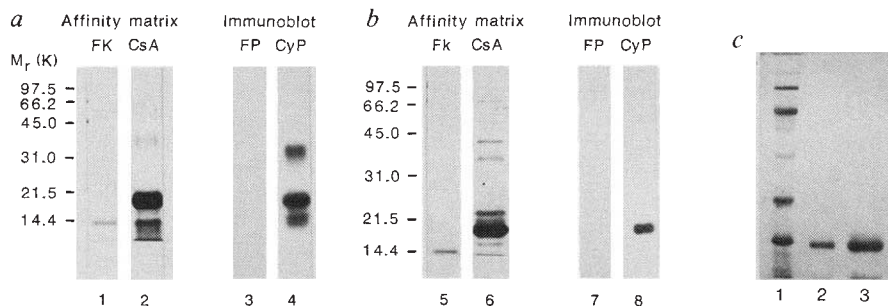


the proteins obtained in this manner with the material eluted from the affinity matrix was determined by N-terminal amino-acid sequencing. The binding of 32 -[1- 14 C]-benzoyl-FK506 (Fig. 1;4) shows a linear dependence on the FKBP concentration (Fig. 3a), but [3 H]-labelled CsA does not bind to this protein

(data not shown). Titration with unlabelled FK506 (Fig. 1;1) displaces the radioligand from its complex in a concentration-dependent manner (Fig. 3b). In a competitive LH-20 binding assay¹⁴, CsA was not displaced from cyclophilin, even by a 500-fold molar excess of FK506. Specificity of FK506 and CsA

FIG. 2 Purification of FK506 and CsA-binding proteins from bovine thymus, *a*, and human spleen, *b*, tissue by affinity adsorption and ligand displacement. Bovine (lane 1) and human (lane 5) FKBP were obtained by elution with FK506. Cyclophilins and other putative CsA-binding or cyclophilin-associated proteins were purified from thymus (lane 2) and spleen (lane 6) by elution with CsA. Bovine (lane 3) and human (lane 7) FKBP do not react with affinity purified rabbit anti-cyclophilin IgG. Bovine (lane 4) and human (lane 8) cyclophilin and a 14K bovine protein react with anti-cyclophilin IgG. The 15, 36 and 40K human proteins (lanes 6 and 8) appear to be antigenically unrelated. *c*, Silver staining of homogeneous bovine FKBP purified by chromatographic methods. Samples of 1 μ g (lane 2) and 2.5 μ g (lane 3) were resolved by SDS-PAGE (12.5% gel).

METHODS. Calf thymus and human spleen tissue were homogenized (1:4 w/v) in 10 mM Tris-HCl (pH 7.4), 120 mM KCl, 5 mM 2-mercaptoethanol and 1 mM PMSF. Homogenates were centrifuged at 8,000g for 20 min, then at 21,000g for 45 min and supernatants were clarified by filtration through a 0.45 μ m membrane. Filtrates were diluted to 2–3 mg protein ml⁻¹ and 45–50 ml passed through 0.5 ml FK506 or CsA Affigel columns. After extensive washing with phosphate-buffered saline–0.05% Tween 20 (50 ml) and then 5 mM Tris-HCl (pH 7.4), 5 mM 2-mercaptoethanol (5 ml), the proteins were recovered by batch elution of the affinity resin with FK506 (500 μ g ml⁻¹), rapamycin (500 μ g ml⁻¹) or CsA (100 μ g ml⁻¹) in a total volume of 1 ml. Approximately 15–30 μ g of drug-binding proteins were recovered in each affinity matrix experiment. Elution fractions were lyophilized, and the proteins were resolved by reducing SDS-PAGE (12.5% gels) and identified by silver staining. Drug additions were made from stock solutions at 10 mg ml⁻¹ in 100% ethanol. Drug concentrations for elution were chosen to equal the concentration of matrix-bound drug. At this concentration, CsA formed a microcrystalline solution which was completely effective at receptor displacement. For purification of drug-free FKBP, clarified tissue homogenates were heated at 60°C for 20 min. Supernatants harvested after centrifugation at 20,000g were concentrated, dialysed against 20 mM Tris-HCl (pH 7.4) and chromatographed on a DEAE Sepharose



column (1.6 cm internal diameter $\times$ 40 cm) equilibrated with dialysis buffer. The void fractions were recovered, concentrated and molecular weight fractions separated on a Sephacryl S100HR column (2.5 cm internal diameter $\times$ 90 cm) equilibrated with 20 mM Tris-HCl (pH 7.4), 150 mM NaCl. The low molecular weight (10–20 K) fraction was recovered and dialysed extensively against 5 mM Tris-HCl (pH 6.9) and the resulting proteins were resolved on a Synchropak CM-300 HPLC column (4.6 mm internal diameter $\times$ 25 cm) by isocratic elution with 5 mM Tris-HCl (pH 6.9), 5 mM NaCl at 1 ml min⁻¹. The FK506 binding activity of resolved proteins was monitored by Sephadex LH-20 partition assay as described⁹ and the FKBP was associated with a single peak with a retention time of 7 min. Homogeneity of human and bovine FKBP was verified by silver staining after SDS-PAGE (panel *c*), isoelectric focusing and N-terminal sequence analysis. This protocol achieved a recovery of approximately 2 μ g FKBP mg⁻¹ of protein in the original clarified tissue homogenates. For immunoblotting, proteins were electrotransferred²² to nitrocellulose and blots were developed with affinity-purified rabbit anti-cyclophilin IgG (1 μ g ml⁻¹) and ¹²⁵I-protein A (2×10^5 c.p.m. ml⁻¹). For sequence analysis, proteins were electroblotted to PVDF membranes as described by Matsudaira²³ and membrane pieces were loaded into the cartridge of an Applied Biosystems Model 477A gas phase sequencer with on-line PTH amino-acid analysis. Initial couplings and repetitive yields of 207 pmol/89% and 21 pmol/87% were calculated for the bovine and human sequences, respectively.

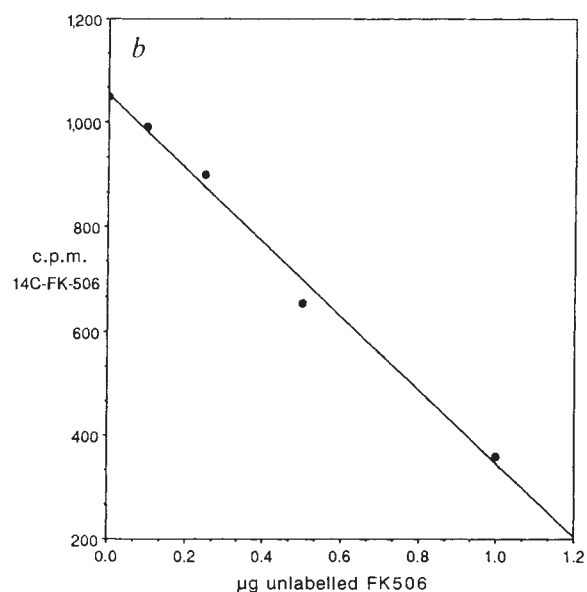
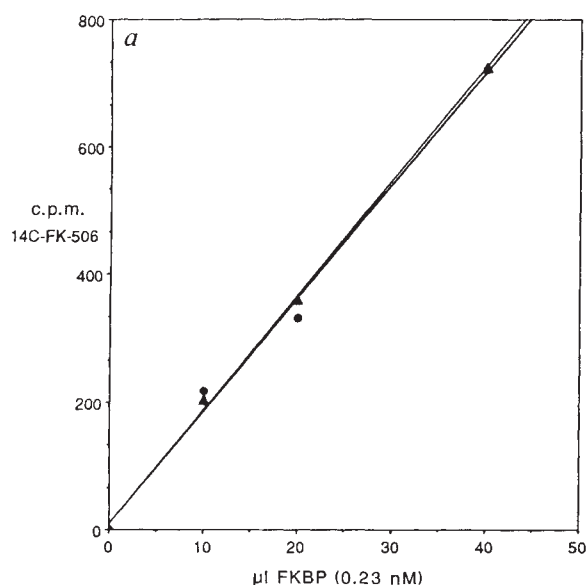


FIG. 3 *a*, Linear binding of 32 -[1- 14 C]-FK506 (Fig. 1;4) to increasing amounts of the bovine FKBP; *b*, titration with unlabelled FK506 (Fig. 1;1). **METHODS.** Increasing amounts of bovine FKBP (63, 126 and 252 ng) were mixed with 1 μ g of 4 (Fig. 1; 2.5×10^5 c.p.m. μ g⁻¹) in a total vol of 100 μ l. FKBP/4 complexes were recovered by a Sephadex LH-20 partition assay⁹.

Titration of FK506 binding to a constant amount of the FKBP was performed by addition of 0.1, 0.25, 0.5 and 1.0 μ g unlabelled FK506 to the 4/FKBP reaction mixture, which resulted in a proportional displacement of radioligand from the complex.

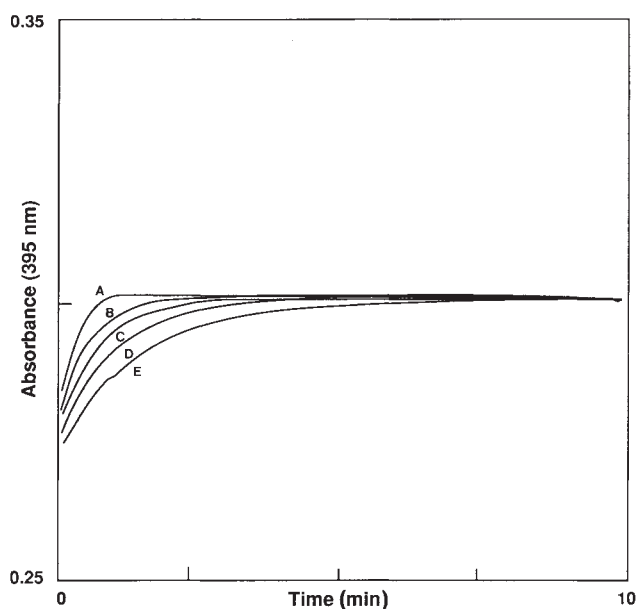


FIG. 4 Isomerase activity of human FKBP and its inhibition by FK506. *Cis-trans* isomerization of succinyl-Ala-Ala-Pro-Phe-4-nitroanilide (27 μ M final concentration) was measured in a coupled assay with chymotrypsin, which hydrolyses the anilide bond in the *trans* (but not *cis*) isomer of the alanyl-prolyl containing peptide¹². The test peptide was preincubated with (a) or without (e) 0.67 nM FKBP at 10°C and the reaction initiated by addition of chymotrypsin (27 μ M final concentration). Reactions were in 100 mM Tris-HCl (pH 8.0) and the hydrolysis of the 4-nitroanilide in the *trans* isomer of the peptide substrate was monitored at 395 nm with a Kontron Uvicon 860 spectrophotometer. FK506 at final concentrations of 27 nM (b), 54 nM (c), and 270 nM (d) resulted in increasing levels of inhibition. Whereas 5 μ M FK506 resulted in complete inhibition of enzyme activity, 5 μ M CsA had no observable effect.

binding was also shown with affinity matrix-purified FKBP and cyclophilin by equilibrium exchange drug-binding assays. Analogously, in affinity matrix-drug elution experiments, the FKBP was not eluted from the FK506 matrix by CsA (100 μ g ml⁻¹) and cyclophilin was not eluted from the CsA matrix by FK506 (500 μ g ml⁻¹). In contrast to these findings, rapamycin (Fig. 1; 5), an immunosuppressive macrolide antibiotic structurally related to FK506 (refs 15,16), readily displaced FKBP from the FK506 affinity matrix, providing further evidence of the specificity of this protein for structures related to FK506.

The similar immunosuppressive properties of FK506 and CsA and the presence of an α -keto (homo)proline amide linkage within the structures of FK506 and rapamycin suggested the possibility that the FKBP could catalyse the interconversion of proline rotamers. Accordingly, the assay of Fischer *et al.*¹² was used to test this hypothesis. In the presence of 0.67 nM human FKBP, the rate of *cis-trans* alanyl-prolyl isomerization of succinyl-Ala-Pro-Phe-4-nitroanilide was significantly increased. In a parallel experiment under identical conditions, a 10–20-fold lower concentration of cyclophilin was sufficient to achieve a comparable level of catalysis¹². Addition of 5 μ M FK506 completely inhibited the isomerase activity of the FKBP, whereas CsA (5 μ M) had no effect. Similarly, FK506 was shown to have no effect on the isomerase activity of cyclophilin (R. E. Handschumacher, personal communication). Experiments with concentrations of FK506 in the range of 27–270 nM showed partial inhibition of the isomerase activity and suggest a K_i of approximately 50 nM (Fig. 4).

The discovery that the CsA receptor cyclophilin functions as an isomerase raised the possibility that the recognition, or perhaps the actual isomerization, or proline-containing epitopes may be relevant to the regulation of intracellular signalling

events in T cell activation^{12,13}. The findings reported here support this proposal. Two immunosuppressive agents with no structural similarity bind to different proteins (immunophilins) that share a common enzymatic property. Each isomerase is inhibited by its respective ligand and these ligands do not exhibit cross inhibition. The apparent structural difference between the FKBP and cyclophilin may reflect the existence of a spectrum of structurally distinct isomerases which may include the *ninaA* gene product^{17,18} and a *Neurospora* mitochondrial protein¹⁹, two recently described cyclophilin homologues. We suggest that proteins of the isomerase (rotamase) class may be components of a biochemical regulatory network that is important for T cell activation and other metabolic processes. □

Note added in proof: Complementary DNA that encodes the FKBP has been cloned from Jurkat cells (R. F. Standaert, G. L. Verdine and S.L.S.). From the deduced amino-acid sequence, the molecular weight of FKBP is calculated to be 11,805.

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1. Tanaka, H. *et al.* *J. Am. chem. Soc.* **109**, 5031–5033 (1987).
2. Kino, T. *et al.* *J. Antibiotics* **40**, 1249–1256 (1987).
3. Thomson, A. W. *Immunology Today* **10**, 6–9 (1989).
4. Sawada, S., Suzuki, G., Kawase, Y. & Takaku, F. *J. Immun.* **139**, 1797–1803 (1987).
5. Yoshimura, N., Matsui, S., Hamashima, T. & Oka, T. *Transplantation* **47**, 351–356 (1989).
6. Yoshimura, N., Matsui, S., Hamashima, T. & Oka, T. *Transplantation* **47**, 356–359 (1989).
7. Sanghvi, A. *et al.* *Transplant. Proc.* **19** (Suppl. 6) **45** (1987).
8. Second International Congress on Cyclosporine. *Transplant. Proc.* **20**, Suppl. 2 (1988).
9. Handschumacher, R. E., Harding, M. W., Rice, J., Drugge, R. J. & Speicher, D. W. *Science* **226**, 544–546 (1984).
10. Harding, M. W., Handschumacher, R. E. & Speicher, D. W. *J. Biol. Chem.* **261**, 8547–8555 (1986).
11. Warti, V. *et al.* *Transplantation* **46**, 453–455 (1988).
12. Fischer, G., Wittmann-Liebold, B., Lang, K., Kiefhaber, T. & Schmid, F. X. *Nature* **337**, 476–478 (1989).
13. Takahashi, N., Hayano, T. & Suzuki, M. *Nature* **337**, 473–475 (1989).
14. Koletsky, A. J., Harding, M. W. & Handschumacher, R. E. *J. Immun.* **137**, 1054–1059 (1986).
15. Findlay, J. A. & Radics, L. *Can. J. Chem.* **58**, 579–590 (1980).
16. Martel, R. J., Klicius, J. & Galet, S. *Can. J. Physiol. Pharmacol.* **55**, 48 (1977).
17. Shieh, B. H., Stamnes, M. A., Seavello, S., Harris, G. L. & Zuker, C. S. *Nature* **338**, 67–70 (1989).
18. Schneuwly, S. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **86**, 5390–5394 (1989).
19. Tropsch, M. *et al.* *J. Biol. Chem.* **263**, 14433–14440 (1988).
20. Coleman, R. S. & Danishefsky, S. J. *Heterocycles* **28**, 157–161 (1989).
21. Bayley, H., Standing, D. & Knowles, J. R. *Tetrahedron Lett.* **39**, 3633–3634 (1978).
22. Gershoni, J. M., Davis, F. E. & Palade, G. E. *Analyt. Biochem.* **144**, 32–40 (1985).
23. Matsudaira, P. *J. Biol. Chem.* **261**, 10035–10038 (1987).

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Characterization of a homologue of bithorax-complex genes in the leech *Hirudo medicinalis*

Joanna W. Wysocka-Diller, Gabriel O. Aisemberg, Miriam Baumgarten, Michael Levine & Eduardo R. Macagno*

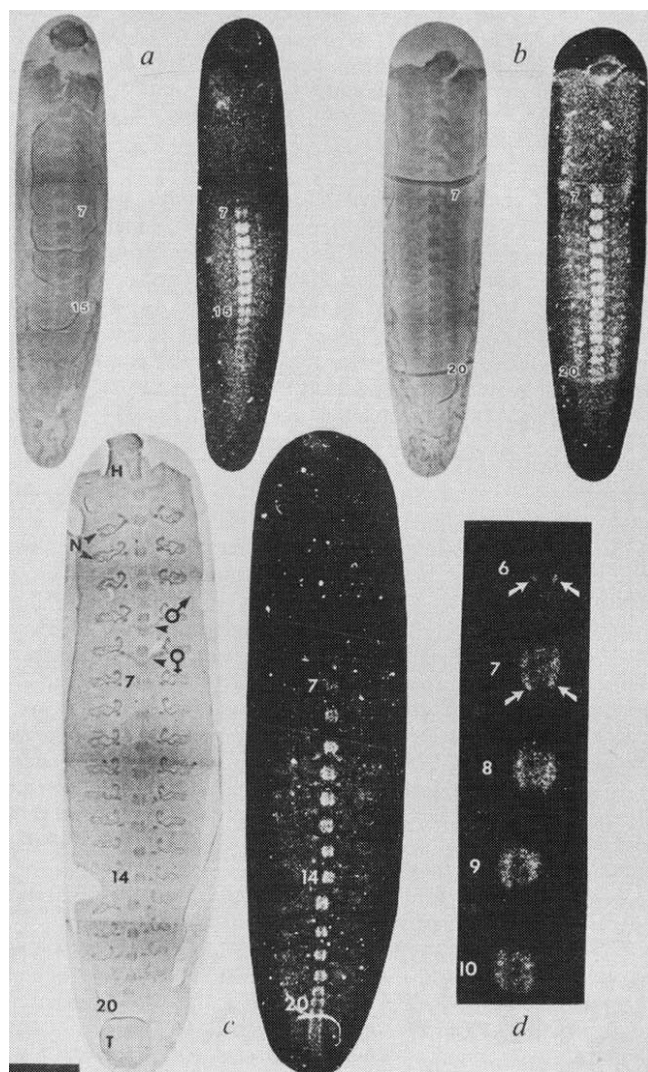
Department of Biological Sciences, Columbia University, New York, New York 10027, USA

WE report the isolation and characterization of the *Hirudo medicinalis* homoeobox gene *Lox2*. Sequence analysis shows that it contains a region that has homology to *Drosophila* and vertebrate homoeodomains of the *Antennapedia* class. In addition, *Lox2* shares homology with sequences in the bithorax complex *Ultra-bithorax* (*Ubx*) and *abdominalA* (*abdA*) genes in a region adjacent to the C-terminus of the homoeodomain. Whole mount *in situ* hybridization of embryos of various ages demonstrates that during early development this gene has temporally and spatially restricted patterns of expression that resemble those of the homoeotic genes

* To whom correspondence should be addressed.

FIG. 2 Localization of *Lox2* transcripts in leech embryos. Dorsal views of whole mounts of embryos of different ages hybridized to ^{35}S -labelled riboprobes. The left side of each panel (a-c) shows a bright-field micrograph of an embryo and the right side its corresponding dark-field micrograph. The numbers on the pictures indicate the position of some of the twenty-one body ganglia. Anterior is at the top. a, Late 8-day-old embryo. The medial region is occupied by the ventral nerve cord, with ganglia that are more differentiated in anterior segments. Some body segments posterior to body segment 16 can be discerned but the tail segments have not yet formed. The highest *Lox2* expression is in the ventral nerve cord from body segment 7 to body segment 15. In this and the following embryos, a diffuse signal can be seen outside the CNS in the segments that express *Lox2* and a weak hybridization is also distinguishable in the primordia of a few, more posterior ganglia. b, A 9-day-old embryo. All body segments are present by this age and the tail segments are just beginning to differentiate. Strong *Lox2* expression is present in body ganglia 7 to 20. c, An 11-day-old embryo. The neuromeres that contribute to the head ganglion (H) have fused, whereas those that make up the tail ganglion (T) are in the process of fusing. The primordia of the male and female genitalia and the bilateral nephridia (N) can be seen. The highest level of *Lox2* transcript accumulation is in the body ganglia 8 to 19. d, Dark-field micrograph showing at higher magnification body ganglia 6 to 10 of a 10-day-old embryo probed with *Lox2*. Arrows indicate posterior cell clusters expressing *Lox2* in ganglia 6 and 7. Clusters in the same position seem to express *Lox2* in more posterior ganglia.

METHODS. Germinal plates and cryptolarvae of dissected leech embryos were fixed in paraformaldehyde, rinsed several times with PBS and permeabilized with pronase¹⁹. The embryos, mounted on polylysine-coated slides, were re-fixed and acetylated²⁰. Finally, slides were rinsed with PBS and dried at room temperature. RNA probes labelled with ^{35}S were synthesized from a plasmid containing a *Lox2* EcoRI-ClaI fragment²¹ (see Fig. 1). A probe with the most 3' 414 nucleotides including only the last quarter of the homoeobox was used with identical results (data not shown). Each embryo was covered with 15 μl of hybridization solution (50% formamide, 10% dextran sulphate, 10 mM DTT, 0.3 M NaCl, 10 mM Tris-HCl, 10 mM sodium phosphate buffer, pH 6.8, 5 mM EDTA, 1x Denhardt's solution and 0.2 mg ml⁻¹ transfer RNA containing $\sim 2 \text{ ng}$ (2×10^5 c.p.m.) of the appropriate riboprobe (adjusted to ~ 100 -bases long by limited alkaline hydrolysis) and incubated overnight at 60 °C. The slides were then washed with 2 changes of low-salt buffer²² for 30 min at 65 °C and treated with RNase A (20 μg ml⁻¹) in NTE buffer (0.5 M NaCl, 10 mM Tris-HCl and 1 mM EDTA) for 1.5 hr at 37 °C. The slides were rinsed again briefly with NTE and washed overnight at 55 °C with several changes of 50% formamide, 2xSSC, 100 mM 2-mercaptoethanol, then rinsed several times in 1xSSC, dried and dipped in Kodak NTB emulsion for autoradiography and exposed for 8 days. After development the embryos were counter stained with Giemsa and mounted with Permount.



its anterior boundary as compared with the dynamic nature of its less sharply defined posterior limit, which shifts caudally at first and then recedes, as the more posterior segments are formed and differentiate. These temporal changes aside, the domain of expression of *Lox2* extends over at least 8 or more segments during the period between 8 and 14 days of development. The embryonic domain of strong *Ubx* expression also changes with time: it is restricted to parasegment (PS) 6 in the cellular blastoderm and in the late embryo, but extends from PS6 to PS12 between these stages; weaker expression in PS5 and PS13 is seen during this time¹¹⁻¹³. Finally, *Lox2* is expressed most strongly in the central nervous system, at least in the embryonic stages we have studied. By contrast, *Ubx* is expressed strongly in epidermal and mesodermal tissues as well as the nervous system, at least in early stages.

The identification of a putative BX-C gene cognate in the leech contradicts an important aspect of previous models for the evolution of the homoeotic genes of the fly^{14,15}. These models are based on the idea that insects are derived from a myriapod-like ancestor with very similar trunk (thoracic+abdominal) segments. They presume that BX-C genes such as *Ubx* and *abdA* arose later in evolution, by the duplication of an *Antp*-like gene, to suppress the action of anterior homoeotic genes within the presumptive abdomen. The finding of *Lox2* in the leech indicates that a BX-C gene homologue was present much earlier than these models propose. As the *Ubx* and *abdA* homoeoboxes are more homologous to each other (56/60 amino-acid identity)

than either of them is to *Lox2*, it is possible that an ancestor of *Lox2* gave rise to both fly genes only after the split of arthropods and annelids. The higher homology found between *Lox2* and *Ubx* also indicates that *Ubx* is the fly gene most closely related to this proposed ancestral gene. Also, it has been proposed that *Antp*, *Ubx* and *abdA* arose from a single gene by duplication during arthropod evolution¹⁵. It would be interesting to know whether there is an *Antp* homologue in the leech in addition to *Lox2*, or whether this gene is the only one that specifies positional information in the central and posterior body segments. □

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- McGinnis, W., Levine, M., Hafen, E., Kuroiwa, A. & Ghering, W. J. *Nature* **308**, 428-433 (1984).
- Scott, M. P., Tamkun, J. W. & Hartzell III, G. W. *Biochim. biophys. Acta Rev. Cancer* **989**, 25-48 (1989).
- Scott, M. P. & Weiner, A. J. *Proc. natn. Acad. Sci. U.S.A.* **81**, 4115-4119 (1984).
- Colberg-Poley, A. M., Voss, S. D., Chowdhury, K. & Gruss, P. *Nature* **314**, 713-718 (1985).
- Schughart, K., Utset, M. F., Awgulewitsch, A. & Ruddle, F. H. *Proc. natn. Acad. Sci. U.S.A.* **85**, 5582-5586 (1988).
- Meijlink, F. et al. *Nucleic Acids Res.* **15**, 6773-6786 (1987).
- Boncinelli, E. et al. *Genome* (in the press).
- Müller, M. M., Carrasco, A. E. & DeRobertis, E. M. *Cell* **39**, 157-162 (1984).
- Mitchell, P. J. & Tjian, R. *Science* **245**, 371-378 (1989).
- Fernández, J. & Stent, G. S. *J. Embryol. exp. Morph.* **72**, 71-96 (1982).
- Akam, M. & Martínez-Arias, A. *EMBO J.* **4**, 1689-1700 (1985).
- Akam, M. *EMBO J.* **2**, 2075-2084 (1983).
- Harding, K., Wedeen, C., McGinnis, W. & Levine, M. *Science* **229**, 1236-1242 (1985).
- Akam, M. *Cell* **57**, 347-349 (1989).
- Lewis, E. B. *Nature* **276**, 565-570 (1978).
- Frischauf, A.-M., Leirach, H., Poustka, A. & Murray, N. *J. molec. Biol.* **170**, 827-842 (1983).

17. Messing, J. & Vieira, J. *Gene* **19**, 269–276 (1982).
18. Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463–5467 (1977).
19. Hafen, E., Levine, M., Garber, R. L. & Gehring, W. J. *EMBO J.* **2**, 617–623 (1983).
20. Angerer, L. M. & Angerer, R. C. *Nucleic Acids Res.* **9**, 2819–2840 (1981).
21. Krieg, P. A. & Melton, D. A. *Methods Enzym.* **155**, 397–415 (1987).
22. Clayton, D. F., Huecas, M. E., Sinclair-Thompson, E. Y., Nastuk, K. L. & Nottebohm, F. *Neuron* **1**, 249–261 (1988).

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Fluorescence energy transfer shows that the four-way DNA junction is a right-handed cross of antiparallel molecules

Alastair I. H. Murchie*, Robert M. Clegg†, Eberhard von Kitzing†‡, Derek R. Duckett*, Stephan Diekmann† & David M. J. Lilley*§

* Department of Biochemistry, The University, Dundee DD1 4HN, UK

† Abteilung Molekulare Biologie, Max-Planck-Institut für Biophysikalische Chemie, D-3400 Göttingen, FRG

THE four-way junction between DNA helices is the central intermediate in recombination^{1–10}, and the manner of its interaction with resolvase enzymes can determine the genetic outcome of the process. A knowledge of its structure is a prerequisite to understanding the interaction with proteins, and there has been recent progress^{11–14}. Here we use fluorescence energy transfer to determine the relative distances between the ends of a small DNA junction, and hence the path of the strands. Our results are consistent with the geometry of an 'X'. The interconnected helices are juxtaposed so that the continuous strands of each helix generate an antiparallel alignment, and the two interchanged strands do not cross at the centre. The acute angle of the X structure is defined by a right-handed rotation of the helical axes about the axis perpendicular to the X plane, as viewed from the centre of the X.

The *in vitro* construction of defined DNA junctions^{11,15–17} has been essential to the understanding of the structure of the four-way junction. The structure introduces a sharp bend in the DNA¹¹. Using a technique related to that of Cooper and Hagerman¹², we studied the configuration of a series of junctions¹³ and proposed a general model for the helical junction—the stacked X structure. The molecular geometry of the X at the junction is formed by the pairwise stacking of the helical arms, resulting in quasi-continuous helices. Helix-helix stacking was first proposed by Sigal and Alberts¹⁸. The symmetry of our structure is consistent with results of hydroxyl radical probing of synthetic junctions¹⁴. The choice of stacking partners is governed by the sequence at the junction, and the pattern of cleavage by phage resolvase enzymes is dependent on the isomeric form adopted for a given DNA sequence^{13,19}. In the absence of metal ions, junctions of any sequence adopt a structure close to square-planar, in which the four arms are unstacked and maximally extended.

Four stereochemical arrangements of the stacked X structure are possible (Fig. 1). Gel electrophoresis experiments indicated that a non-crossed structure was correct¹³, but this conclusion rested on assumptions about the electrophoretic mobility of DNA. Here we have used an independent approach, employing fluorescence energy transfer techniques to measure relative end-to-end distances in the junction. The stacked X-model predicts that two of the end-to-end distances from the possible six will

be shorter than the others, and identification of these will distinguish between crossed and non-crossed structures. Two fluorescent dyes attached to a macromolecule can exhibit an overlap of emission (donor) and excitation (acceptor) frequencies, when energy transfer then becomes possible from donor to acceptor. The transfer is dependent on the distance between the donor and acceptor (in the size range from 30 to 70 Å); the efficiency of transfer (E) is given by $E \propto (1 + (R/R_0)^6)^{-1}$, where R is the distance between the dyes, and R_0 is the distance where the efficiency of transfer is 0.5 (refs 20, 21).

We attached fluorescein and tetramethyl rhodamine to the 5' ends of selected oligonucleotides used for the assembly of junctions to investigate the energy transfer across the six possible end-to-end distances, each of which could be studied in both directions. These experiments were carried out for a variety of arm lengths, but the best results were obtained using junctions constructed from oligonucleotides of 34 bases, giving junctions of arm length 17 base pairs (see below). As the strength of a dipole-dipole interaction is dependent on orientation as well as distance, at least one of the dyes had to be relatively mobile. Hence we used a C_6 linker to attach the dye to the DNA. Subsequent measurements of fluorescence anisotropy indicated that there was some interaction between the dyes and the ends of the arms, so we included a 5' CpC on each arm to maintain a constant environment for the dyes. As we can unfold the junction by the removal of ions¹³, the energy transfer can be measured before and after the addition of magnesium while keeping all other factors constant.

The junction sequences chosen for these studies were essentially those of junctions 1 and 2 (ref. 13), previously shown to have different isomeric conformations. The central sequences are given in Fig. 2a. The ratios of fluorescence energy transfer for junctions 1 and 2 with and without the addition of 5 mM magnesium ions are shown in Fig. 2b. For junction 2, the largest increase in energy transfer upon addition of Mg^{2+} was for the doubly labelled arms B–H or R–X. By contrast, the results for junction 1, with the opposite isomeric configuration¹³, show the largest increase for the B–X and H–R labelled arms (Fig. 2b). Both these results verify the conclusions of our earlier gel electrophoresis experiments¹³. In addition, the relative energy

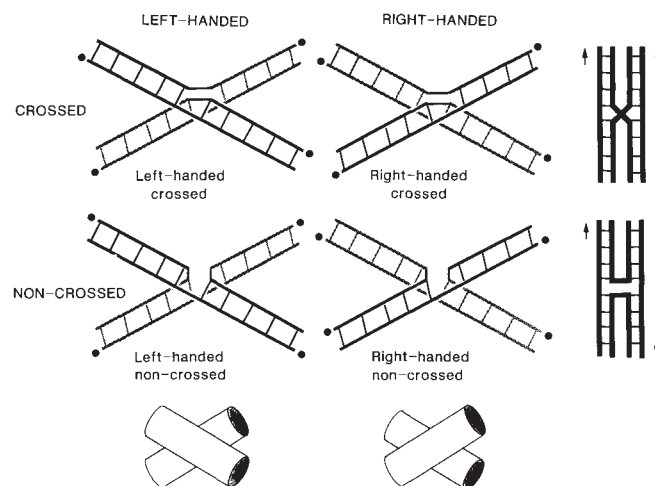
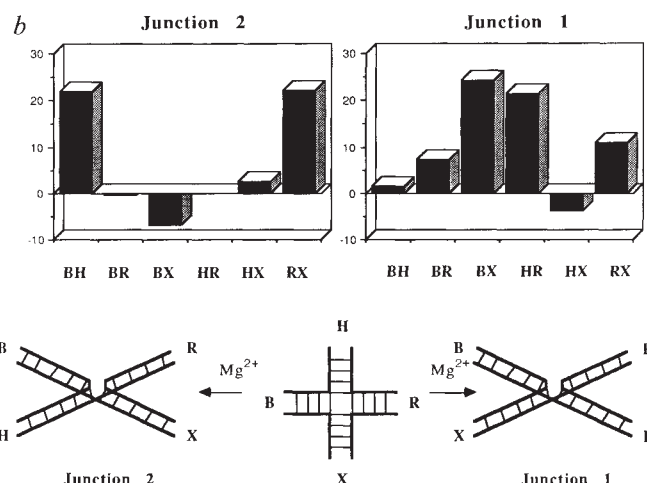


FIG. 1 Possible topology of strands and helices in the stacked X structure of the four-way junction. The strands can be drawn as non-crossed or crossed, generating an antiparallel or parallel alignment of the sequences respectively (indicated by the arrows). Because of the non-planarity of the X, the helices can cross in either a right-handed or a left-handed form (the convention for the handedness indicated is based on the definition of dihedral angles²⁴). The helices at the back are stippled. The combination of these variables generates four possible structures. The 5' termini of strands are indicated by filled circles, and are designated in such a way that all interconversions can be carried out in the plane of the page.

§ Present address: Max-Planck-Institut für Medizinische Forschung, Abteilung Zellphysiologie, Postfach 103820, D-6900 Heidelberg, FRG.

The stereochemical analysis predicts that the stacked X structure should be right-handed. Inspection of the right-handed, non-crossed model for junction 2 reveals that the phosphate situated 18 bases from the junction on the 5' strand of arm B is closest to the H arm. We therefore prepared a series of labelled junctions in which fluorescein was attached to this position on arm B. In this series, we varied the length of the H arm (while maintaining the 5' CpC sequence) between 12 and 21 base pairs,



The ratio $R_{\frac{490/600}{565/600}}$ is constant and only involves the absorption coefficients. As this is relatively small, R measures the ratio of the fluorescence due to energy transfer to the direct fluorescence of rhodamine. These values were measured in the presence and absence of magnesium ions, and their difference is presented graphically. When this was measured for a doubly labelled isomer with both permutations of labels, an average is shown. As there is a linear relation between the absorption and fluorescence of rhodamine for all samples, this ratio normalizes the extent of transfer for all the samples, and the ratio is proportional to the per cent transfer. Only the optical constants of the dyes determine the proportionality constant. The schematic drawings below the graphs for junctions 2 and 1 illustrate the interpretation of the fluorescent energy transfer changes. For junction 2, the distances that shorten on magnesium-induced

folding, causing increased transfer, were B-H and R-X, indicating a non-crossed structure with co-linear B on X and H on R arms. For junction 1, the shortened distances are B-X and H-R, indicating a non-crossed structure with co-linear B on H and R on X arms.

METHODS. Preparation of dye-conjugated junctions: Oligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer using β -cyanoethyl phosphoramidite chemistry^{25,26}. A C_6 linker with a free amine group was incorporated at the 5' end of the molecule by a final coupling with *N*-trifluoroacetyl-2-aminohexyl(2-methoxy)-*N,N*-diisopropylamino phosphite²⁷ (Applied Biosystems). Crude oligonucleotides were purified on an anion exchange column (Nucleogen, Diagen), followed by a C_8 reverse-phase column (Aquapore, Applied Biosystems). Each oligonucleotide (one absorbance unit at 258 nm) was reacted with 3 μ l of 50 mg ml⁻¹ of the *N*-hydroxy-succinimide ester of tetramethylrhodamine (Applied Biosystems) in dimethylsulphoxide with 0.2 M carbonate buffer (pH 9.5) to give a total volume of 15 μ l for 4–12 h at room temperature in the dark, and, in a separate reaction, with 20 μ l of 1.0 mg ml⁻¹ fluorescein isothiocyanate (Sigma) in dimethylformamide with 0.3 M carbonate buffer (pH 9.5) for 4–12 h at room temperature in the dark. After separation of unreacted dyes on Sephadex G25, conjugated oligonucleotides were purified by electrophoresis in a 20% polyacrylamide gel containing 7 M urea. In order to construct junctions, oligonucleotide solutions (0.04 absorbance units at 258 nm) were hybridized in appropriate combinations in 2 mM MgCl₂, 3 $\times$ SSC at 65°C, and slowly cooled to 10°C. Junctions were purified by electrophoresis in 8% polyacrylamide gels in 90 mM Tris-borate (pH 8.3), 2 mM MgCl₂, with circulating buffer. Junctions were excised, electroeluted and ethanol-precipitated.

Fluorescence spectroscopy: Fluorescence spectra were recorded using an SLM 8000S fluorimeter. Excitation and emission spectra were corrected for lamp variation and instrumentation factors, and polarization artefacts were eliminated using magic angle conditions. Data were acquired and analysed with a DEC-LSI/23 computer. Dye concentrations were generally between 50 and 150 nM; per cent labelling was estimated from absorption spectra to be constant at 100% for all the samples. The absorption spectra of fluorescein, rhodamine and DNA were linearly related among all the samples and the excitation spectrum maxima that were due only to directly excited rhodamine were linearly related to the corresponding absorption spectra. The assembled dye-labelled junctions were dissolved in 90 mM Tris-borate (pH 8.3), 0.1 mM EDTA, for spectroscopy; conditions were identical to those used in gel electrophoresis experiments¹³. Magnesium chloride was added from a concentrated stock solution.

and attached rhodamine to the 5' ends. This has the effect, shown diagrammatically in Fig. 4a, of 'walking' the rhodamine dye around the H arm helix. Consideration of the right-handed model suggests that the two dyes should be closest when the rhodamine is attached to base 16, so energy transfer should exhibit a maximum for this junction. For a left-handed cross the situation is reversed, with these phosphates located on opposite sides of the helix. The results are presented graphically in Fig. 4b. The energy transfer exhibited a maximum when the rhodamine was attached to phosphates 15 to 16, in agreement with the prediction based on the right-handed structure. We therefore conclude that the four-way junction is indeed a right-handed, non-crossed structure.

A number of biological implications follow from the structure we have deduced for the four-way junction. Geneticists are accustomed to drawing the Holliday junction with the homologous DNA molecules aligned in a parallel manner, but this does not seem to be the natural energetic preference of DNA. Accessory proteins may influence the structure of recombination intermediates in the cell, but if that is so, then we have identified an important role for them. Antiparallel Holliday junctions would have consequences for branch migration. The 60° cross implies that the point of contact between homologous DNA molecules is limited to a very small region around the junction. Antiparallel junctions complicate synapsis between homologous sequences, but not fatally, given the long-range flexibility of DNA molecules. Antiparallel orientation might simplify certain site-specific recombination processes: for example, the topology of integration and excision of phage λ becomes symmetrical in both directions if the junction is drawn in the non-crossed form²³. As physical information is available

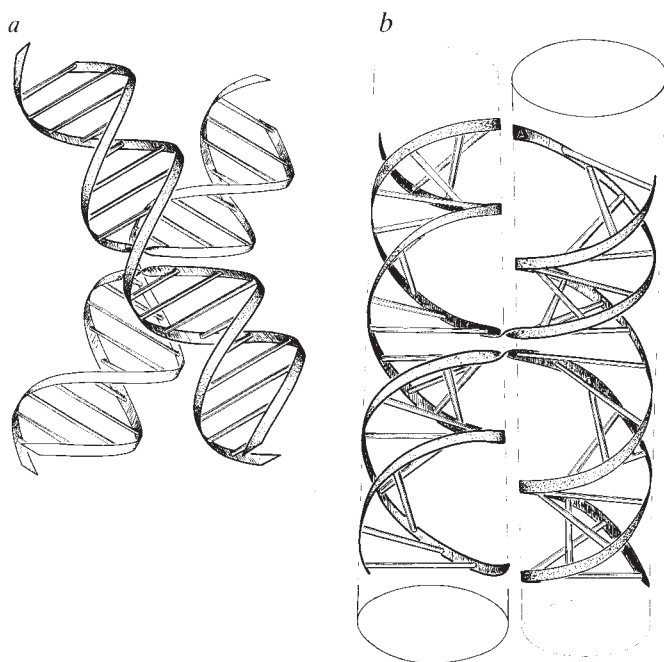


FIG. 3 A non-crossed, right-handed structure avoids unfavourable phosphate-phosphate interactions. *a* and *b*, Front and side views of the structure. In this relative orientation of the two quasi-continuous helices, it can be seen that the continuous strands are accommodated in the major groove of the opposite helix, at the position closest to the centre of the junction. This point may be appreciated most readily by examination of two simple DNA models inclined correctly. Molecular mechanics calculations (E.v.K., D.M.J.L. and S.D., manuscript in preparation) indicate that the non-crossed right-handed structure does not violate stereochemical principles, and a related packing of helices has recently been found in a crystal of a double-stranded DNA oligonucleotide (D. Moras, personal communication).

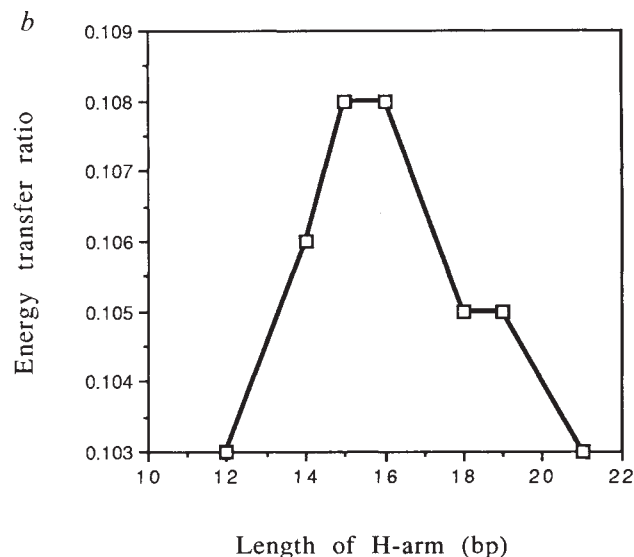
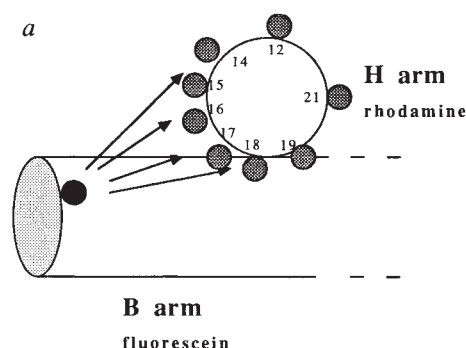


FIG. 4 Fluorescence energy transfer indicates that the helices cross in a right-handed manner. Junctions based on the central sequence of junction 2 were constructed with arm lengths of B (18 base pairs), H (12 to 21 base pairs), R and X (both 15 base pairs). These were prepared with dye-conjugated oligonucleotides as before, with fluorescein attached to the 5' end of the B arm, and rhodamine on the H arm. *a*, Schematic to illustrate the relative dispositions of the B arm and H arm dyes for a right-handed cross. Model building indicates that the smallest dye-dye distance should occur when the H arm is ~16 base pairs. *b*, Energy transfer as a function of the position of rhodamine attachment. Plot is of fluorescence transfer ratio against the length of the H arm, for the junctions in the presence of 5 mM magnesium ion. The transfer is clearly maximal for an H-arm length of 15-16 base pairs, in agreement with the prediction of a right-handed X structure. Equivalent results were obtained (data not shown) for the reverse direction, $H_{\text{(fluorescein)}} - B_{\text{(rhodamine)}}$.

directly from fluorescence energy transfer experiments on nucleic acids, the technique might be applied profitably to related systems, such as the analysis of curved DNA in solution. □

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- Holliday, R. *Genet. Res.* **5**, 282-304 (1964).
- Meselson, M. S. & Radding, C. M. *Proc. natn. Acad. Sci. U.S.A.* **72**, 358-361 (1975).
- Broker, T. R. & Lehman, I. R. *J. molec. Biol.* **60**, 131-149 (1971).
- Sobell, H. M. *Proc. natn. Acad. Sci. U.S.A.* **69**, 2483-2487 (1972).
- Sobell, H. M. in *Mechanisms in Recombination* (ed. Grell, R. F.) 433-438 (Plenum, New York, 1974).
- Orr-Weaver, T. L., Szostak, J. W. & Rothstein, R. J. *Proc. natn. Acad. Sci. U.S.A.* **78**, 6354-6358 (1981).
- Kitts, P. A. & Nash, H. A. *Nature* **329**, 346-348 (1987).
- Nunes-Duby, S. E., Matsumoto, L. & Landy, A. *Cell* **50**, 779-788 (1987).
- Hoess, R., Wierzbicki, A. & Abremski, K. *Proc. natn. Acad. Sci. U.S.A.* **84**, 6840-6844 (1987).
- Jayaram, M., Crain, K. L., Parsons, R. L. & Hershey, R. M. *Proc. natn. Acad. Sci. U.S.A.* **85**, 7902-7906 (1988).
- Gough, G. W. & Lilley, D. M. J. *Nature* **313**, 154-156 (1985).
- Cooper, J. P. & Hagerman, P. J. *J. molec. Biol.* **198**, 711-719 (1987).

13. Duckett, D. R. *et al. Cell* **55**, 79–89 (1988).
14. Churchill, M. E. A., Tullius, T. D., Kallenbach, N. R. & Seeman, N. C. *Proc. natn. Acad. Sci. U.S.A.* **85**, 4653–4646 (1988).
15. Bell, L. R. & Byers, B. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3445–3449 (1979).
16. Kallenbach, N. R., Ma, R.-I. & Seeman, N. C. *Nature* **305**, 829–831 (1983).
17. Hsu, P. L. & Landy, A. *Nature* **311**, 721–726 (1984).
18. Sigal, N. & Alberts, B. *J. molec. Biol.* **71**, 789–793 (1972).
19. Mueller, J. E., Kemper, B., Cunningham, R. P., Kallenbach, N. R. & Seeman, N. C. *Proc. natn. Acad. Sci. U.S.A.* **85**, 9441–9445 (1988).
20. Förster, T. *Ann. Phys.* **2**, 55–75 (1948).
21. Förster, T. *Fluorescence Organischer Verbindungen* (Vandenhoeck & Ruprecht, Göttingen, 1951).
22. Fairclough, R. H. & Cantor, C. R. *Meth. Enzym.* **48**, 347–379 (1978).
23. Stark, M., Sherratt, D. & Boocock, M. *Cell* **58**, 779–790 (1989).
24. Klyne, W. & Prelog, V. *Experientia* **16**, 521–523 (1960).
25. Sinha, N. D., Biernat, J., McManus, J. & Koster, H. *Nucleic Acids Res.* **12**, 4539–4557 (1984).
26. Beaucage, S. I. & Caruthers, M. H. *Tetrahedron Lett.* **22**, 1859–1862 (1981).
27. Coull, J., Wright, L. & Bischoff, F. *Tetrahedron Lett.* **27**, 3991–3995 (1986).

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CORRECTION

Correlation between the anaesthetic effect of halothane and saturable binding in brain

A.S. Evers, B.A. Berkowitz & D. A. d'Avignon

Nature **328**, 157–160 (1987).

In a previous issue of *Nature*, we reported that the anaesthetic effect of halothane correlated with the occupancy of a saturable chemical environment in rat brain¹. We have extended these observations by examining the equilibration of halothane between cerebral cortical brain slices and anaesthetic-equilibrated buffer, an experimental paradigm in which whole-animal physiology cannot influence brain anaesthetic uptake. These studies showed that brain-slice halothane concentration was, with only minor deviation, a linear function of buffer concentration. This discrepancy between *in vitro* and *in vivo* equilibration studies led us to re-examine *in vivo* uptake of anaesthetic by simultaneously measuring brain and blood halothane concentrations in rats anaesthetized with various inspired concentrations of halothane. These studies showed higher brain and plasma halothane concentrations than those we originally reported (Fig. 1): this results from improved calibration technique and possibly, in plasma, from reduced loss of halothane by volatilization during sample preparation. Brain and blood halothane concentrations were seen to rise in parallel as a function of inspired concentration, and the brain/blood halothane ratio (inset of Fig. 1) was constant over the entire inspired concentration range studied. The observed nonlinear relationship between inspired and blood halothane concentrations was not the result of blood saturation, as *in vitro* halothane/gas partition coefficients (37 °C) were the same (2.78 ± 0.08) at 1% (v/v) and 3% halothane. These data indicate that anaesthetic-induced depression of halothane uptake into blood is predominantly responsible for the apparent saturation of brain observed *in vivo*; the apparent saturation reported earlier¹ is not the result of a limited number of binding sites in brain.

We have also reported the existence of two chemical environments for halothane (and for other volatile anaesthetics) in brain², as defined by ¹⁹F-NMR spin-spin relaxation behaviour¹. The two environments are differentially occupied as a function of both inspired anaesthetic concentration and duration of

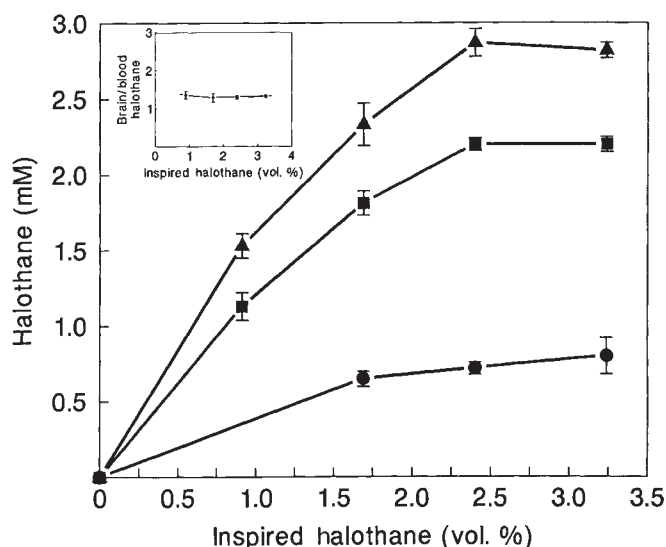


FIG. 1 Brain and blood halothane concentrations simultaneously measured in spontaneously ventilating rats. Rats were allowed to breathe various inspired concentrations of halothane (measured by gas chromatography) for 1 hour. Arterial blood was withdrawn and the animals were decapitated. Blood halothane concentrations (■) were measured by gas chromatography, and brain halothane concentrations (▲) were measured both by gas chromatography and by NMR (ref. 1). Gas chromatography and NMR measurements of brain halothane gave identical results. In parallel experiments, plasma halothane concentrations (●) were measured by gas chromatography. Note that plasma halothane concentrations were reasonably well predicted by our *in vitro* measurement of the rat plasma/gas partition coefficient of ~ 1.3 . The inset shows the brain/blood halothane concentration ratio plotted as a function of inspired concentration. All data are the mean $\pm$ s.d. of 3–6 determinations.

delivery; occupancy of one of these environments (that with a short T_2) correlates with the anaesthetic effect of halothane. In light of our recent findings, it is apparent that occupancy by halothane of the short T_2 environment scales with total brain concentration; this environment is not saturable over the range of concentrations attainable *in vivo*. The other (long T_2) halothane environment represents an increasing proportion of total brain halothane concentration as a function of increasing inspired concentration and duration of anaesthetic administration. The basis for differential occupancy of the two halothane environments is not known, but could be the result of time- and concentration-dependence of either anaesthetic-induced membrane disruption³ (producing the long T_2) or changes in regional blood-flow distribution (for example, to white and grey matter). Although the physical chemical basis for the ¹⁹F-NMR relaxation behaviour of anaesthetics in brain remains unknown, this relaxation behaviour is likely to be pharmacologically relevant, as evidenced by the strong correlation between volatile anaesthetic potency and ¹⁹F-NMR T_2 values in brain². We are continuing to investigate the structural basis for and the pharmacological relevance of anaesthetic ¹⁹F relaxation behaviour in brain. This letter is submitted to correct the record as regards our previous report¹. □

1. Evers, A. S., Berkowitz, B. A. & D. A. d'Avignon *Nature* **328**, 157–160 (1987).

2. Evers, A. S., Haycock, J. C. & D. A. d'Avignon *Biochem. Biophys. Res. Commun.* **151**, 1039–1045 (1988).

3. Rottenberg, B., Waring, A. & E. Rubin *Science* **213**, 583–589 (1981).

Computers in behavioural research

L. P. J. J. Noldus, E. L. H. M. van de Loo and P. H. A. Timmers

Event recording methods have evolved from stopwatches and checksheets, through chart recorders and dedicated electronic event recorders, to flexible systems based on off-the-shelf microcomputers.

MANY types of behavioural research, in disciplines ranging from ethology and behavioural ecology to pharmacology, toxicology, psychology and psychiatry, require accurate timing and recording of sequences of events. For a long time, the most common recording method consisted of timing with a stopwatch in combination with hand-written notes on a checksheet. Mechanical chart recorders, introduced in the 1960s, allowed more accurate timing of rapidly occurring streams of events. Consequently, observations could be of greater complexity with regard to the number of different events and subjects observed. However, as both methods involve considerable manual data processing, there exists an ongoing interest in electronic event recording systems. These offer two major advantages over earlier methods: they save time and they reduce the occurrence of errors because data do not have to be transcribed.

Over the past two decades, several types of electronic event recorders have been developed. These typically consist of a specially designed keyboard with a number of pushbuttons and/or switches, the state of which is internally scanned at very short time intervals. Most early types of event recorders encode observations on magnetic tape for later storage and analysis on a mini or mainframe computer. More recent types have battery-powered electronic memory, which allows operation away from a permanent data storage device and, thus, increases portability. Several event recorders have become commercially available, for example, the Datamyte 900 (Electro/General Corp., Minnetonka, Minnesota)¹, the SSR System 7 (Semeiotic Systems Corp., Madison, Wisconsin)², the OS-3 (Gagetalk Corp., Bellevue, Washington) and The Assistant (Human Technologies, Inc., St Petersburg, Florida)³.

Although these recorders are well adapted to the task for which they were designed, each has its limitations. All of them are dedicated systems. Further, commercially available models are expensive, while others have to be assembled from electronic circuits and, therefore, require technical expertise and considerable input of labour. The maintenance of such equipment also requires specialized personnel.

Use of microcomputers

Commercially available microcomputers that allow data to be entered directly into

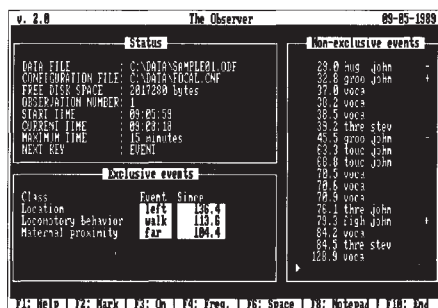


FIG. 1 Screen of a personal computer during an observational data collection session.

the computer render custom-designed keyboards largely unnecessary. Moreover, one can rely on dealer networks for maintenance. Perhaps most important of all, is the great flexibility offered by microcomputers — they can be programmed more easily, in high-level languages such as BASIC, Pascal and C. Most are equipped with a printer port, allowing a hard copy of collected data directly after an observation session, and a reasonably sized screen, allowing direct visual feedback during operation. Interaction via the screen also helps alleviate the concern about the 'invisibility' of computer-collected data. Most of these options are not available with dedicated event recorders.

Today's researcher can choose from a range of different computers for behavioural observations. In fact, any computer that is equipped with a real-time clock can be used as an event recorder. With the aid of alternative input devices — contact sensors and infrared detectors — connected to a communication port or an add-in board, a computer can be used as an activity monitor. In observational research, the type of computer selected depends on various aspects of the study, such as experimental design and location of observations. For instance, complex observations in a laboratory environment may benefit most from a powerful desktop PC, while field studies require a small portable device. As far as permanent data storage and analysis are concerned, today's PCs are powerful enough to perform most of the tasks that required a minicomputer less than a decade ago. Thus, in some circumstances the same device can be used for event recording and data analysis. However, where no direct hardware-compatibility exists between the apparatus on which data are collected and that on which they are to be analysed, a data transfer medium is also necessary.

With computers becoming more powerful, more compact and relatively less expensive, computer event recorders can be applied in an increasing number of different research settings. There are, however, minimum size requirements for direct data entry via a keyboard. For instance, the Psion Organiser II (Psion PLC, London) can store 320 Kbyte and yet weighs only 250 g, but its keys are so small that it does not appear very practical for use as an event recorder.

The accuracy of computerized event recording through manual data entry ultimately depends on the human observer. Most systems time events through the computer clock, which implies that user errors with regard to timing cannot be fixed exactly. If precise timing is at a premium, one solution is to make a video recording and store the real time on the audio track of the video tape. With a specific hardware interface and appropriate software the complete behavioural record can then be edited afterwards at the level of single video frames with the original times still available. Such systems also allow subsequent passes through a video recording for coding of multiple events to a common time base⁴ (Mr. R. Kruk, personal communication).

Event-recording software

As computers replace each other in rapid succession, the availability of software becomes the limiting factor in the widespread use of computer event recorders. An event-recording program should be adapted to the machine on which it has to be run, as well as to the experimental design for which it will be used. The flexibility of microcomputers and high-level programming languages facilitates programs with, for example, trapping and correction of user input errors, distinction between frequency versus duration events, mutually exclusive versus non-exclusive events, and storage of independent variables. However, the use of compact portable computers with limited memory and processing speed restricts the size and level of sophistication of event-recording programs.

Event-recording software has been developed for several commercial microcomputers. Some of these programs have been designed for data collection and analysis on a desktop computer, such as the Apple II⁵, Macintosh⁶ or IBM PC⁷. Most programs operate on a specific combination of a small portable computer for

data collection and a larger computer for analysis, for example, the TRS-80 Model 100/TRS-80 Model 4⁸ or Epson PX-8/BBC Model B⁹. Other systems use the IBM PC as a host computer with, for example, a NEC PC-8201A, PC-8300¹⁰, or Epson HX-20¹¹ as an event recorder. Data-collection software has even been developed for HP41 and HP71 pocketcomputers¹². However, with the current short lifetime of computer models, specific programs written for a particular type of computer run the risk of soon becoming obsolete.

As most behavioural scientists lack programming expertise, there is a need for general-purpose event recording and data analysis software. Such software should (1) be very flexible with regard to hardware and experimental design, (2) be intuitively understandable and require no programming by the user, (3) be robust against user errors, and (4) produce data in a format compatible with commercially available analysis software.

The Observer (Fig. 1), a recently developed software package for observational research¹³, represents an attempt in this direction. This program runs on any IBM-type PC. The user can configure the event recorder accurately to many different experimental designs. With the information entered by the user, the PC can be used directly as an event recorder. In addition, four types of non-IBM compatible portable computers are supported as event recorders: TRS-80 Model 100, Tandy 102, Olivetti M10, and Epson PX-8. For these computers, The Observer generates event-recording programs specifically adapted to the machine and experimental design chosen by the user. As the programs contain only code essential to the current task, they remain compact and occupy a minimum amount of memory in the portable computer. The program transfers generated programs to the portable event recorder and retrieves collected data files. The Observer also analyses observational data and provides the user with statistics in printed reports.

The trend in software is towards computer-user interaction at an increasingly higher level. Ideally, the user should merely have to specify what the problem is, not how it should be solved. The development of such high-level software opens new possibilities for computer-aided behavioural research. With the arrival of computer programs that do the programming, the behavioural scientist can concentrate better on the actual research. □

Lucas P. J. J. Noldus is at the Dept. of Entomology, Agricultural Univ. Wageningen, The Netherlands. Erik L. H. M. van de Loo is at the Dept. of Clinical and Health Psychology, Univ. of Leiden, and the Inst. for Human Resource Management, The Netherlands. Paul H. A. Timmers is at Philips Data Systems BV, Apeldoorn, The Netherlands. For more information, fill in the reader service number 100.

New tools for neuroscience

Exhibits at next week's American Society for Neuroscience Annual Meeting in Phoenix, Arizona include eicosanoid EIA kits and an activity monitor.

THREE new **eicosanoid enzyme immunoassay kits** for the measurement of prostaglandin E₂, thromboxane B₂ and 6-keto-prostaglandin F_{1α} are available from Advanced Magnetics, Inc. (*Reader Service No. 101*). These solution-phase assays are based on the competition of variable amounts of analyte with a fixed amount of alkaline phosphatase-labelled analyte for a limited number of rabbit antibody binding sites. The addition of magnetic goat anti-rabbit antibody, followed by magnetic separation or centrifugation, separates antibody-bound from unbound analyte. The resulting pellet is reacted with para-nitrophenyl phosphate substrate and the absorbance read at 405 nm: sample concentration is determined from a standard curve. AMI says, the prostaglandin E₂, thromboxane B₂ and 6-keto-prostaglandin F_{1α} can detect 0.13 pg, 4.0 pg and 0.12 pg per 0.1 ml sample, respectively. The \$295 (US) kits contain enough reagents for 100 assay tubes, including standard curves, says AMI, who will be in booth 425.

Data acquisition

HVS Image will be exhibiting, in booth 2011, their VP118 Super Track Unit **activity monitor** for monitoring the behavioural activity of animals, particularly rodents (*Reader Service No. 102*). The VP118 receives input from a video camera positioned over an open field, water or radial maze. Positional information from the image is extracted by the VP118 and relayed to the user's IBM PC/XT/AT. Super-Track software analyses data for path length, positional preference, and percentage time spent moving. The system can track two animals together, which is useful for studying social interaction, and can also measure rota-

tional activity. In quad mode, the VP118 can track up to four trials at once.

World Precision Instruments, Inc., exhibiting in booths 923-927, will be showing off the PolyGraf/8 data acquisition system, which provides real-time 'gapless' multichannel **data collection, display and storage** in the form of a data logger or scrolling strip chart recorder (*Reader Service No. 103*). The \$1,695 (US) PolyGraf/8 system includes a plug-in data acquisition card for a MacII, IIx or IIcx, providing eight channels of single-sided bipolar input, a BNC signal manifold, user-friendly software, and cable assembly. Special features include storage of data files and data display during acquisition and in playback mode, adjustable forward and reverse scrolling speed for visual tracking in playback mode, and multiple channel overlay. With PolyGraf/8, the user can 'mark' data points and take snapshots of points of interest for incorporation into word processing documents. □

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1. Torgerson, L. *Behav. Res. Meth. Instrum.* **9**, 405-406 (1977).
2. Stephenson, G.R. & Roberts, T.W. *Behav. Res. Meth. Instrum.* **9**, 434-441 (1977).
3. Paggeot, B., Kvale, S., Mace, F.C. & Sharkey, R.W. *J. appl. Behav. Anal.* **21**, 429 (1988).
4. Krauss, R.M., Morrel-Samuels, P. & Hochberg, J. *Behav. Res. Meth. Instrum. Comp.* **20**, 37-40 (1988).
5. Flowers, J.H. *Behav. Res. Meth. Instrum.* **14**, 241-249 (1982).
6. Deni, R. *Perc. Mot. Skills* **65**, 398 (1987).
7. EventLog (Conduit, Univ. of Iowa, Iowa City, Iowa).
8. Deni, R., Szijarto, K., Eisler, A. & Fantauzzo, C. *Behav. Res. Meth. Instrum. Comp.* **15**, 616 (1983).
9. Unwin, D.M. & Martin, P. *Behaviour* **101**, 87-100 (1987).
10. Juniper Gardens Children's Project, Kansas City, Kansas: S & K Computer Products, Toronto, Ontario.
11. PCS (Communitytech International, Wheaton, Illinois).
12. Whiten, A. & Barton, R.A. *Trends Ecol. Evol.* **3**, 146-148 (1988).
13. Noldus, L.P.J.J. *Abstr. 1st Eur. Congr. Psychol. (Amsterdam)*, 567 (1989), and *Abstr. 21st int. Ethol. Conf. (Utrecht)*, 126 (1989).

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